7168–7180 *Nucleic Acids Research, 2008, Vol. 36, No. 22* *Published online 10 November 2008*
doi:10.1093/nar/gkn888

Induction of *PPM1D* following DNA-damaging treatments through a conserved p53 response element coincides with a shift in the use of transcription initiation sites

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Received January 14, 2008; Revised September 14, 2008; Accepted October 20, 2008

ABSTRACT

PPM1D (Wip1), a type PP2C phosphatase, is expressed at low levels in most normal tissues but is overexpressed in several types of cancers. In cells containing wild-type p53, the levels of *PPM1D* mRNA and protein increase following exposure to genotoxic stress, but the mechanism of regulation by p53 was unknown. *PPM1D* also has been identified as a CREB-regulated gene due to the presence of a cyclic AMP response element (CRE) in the promoter. Transient transfection and chromatin immunoprecipitation experiments in HCT116 cells were used to characterize a conserved p53 response element located in the 5′ untranslated region (UTR) of the *PPM1D* gene that is required for the p53-dependent induction of transcription from the human *PPM1D* promoter. CREB binding to the CRE contributes to the regulation of basal expression of *PPM1D* and directs transcription initiation at upstream sites. Following exposure to ultraviolet (UV) or ionizing radiation, the abundance of transcripts with short 5′ UTRs increased in cells containing wild-type p53, indicating increased utilization of downstream transcription initiation sites. In cells containing wild-type p53, exposure to UV resulted in increased PPM1D protein levels even when *PPM1D* mRNA levels remained constant, indicating post-transcriptional regulation of PPM1D protein levels.

INTRODUCTION

PPM1D, a member of the PP2C family of serine/threonine protein phosphatases, was first identified as a wild-type p53-induced phosphatase (Wip1), the expression of which was increased in human cells after ionizing radiation (IR) or exposure to ultraviolet (UV) light (1). The human PPM1D and mouse Ppm1d proteins are 83% homologous and are similarly induced in response to stress (2). *PPM1D* transcription is induced following exposure to many DNA damage-inducing agents, including NO (3), H_2O_2 (4) and MNNG (5). *PPM1D* was identified as a component of a 16-gene signature characterizing the p53-dependent DNA-damage response (6). The transient increase in *PPM1D* mRNA levels following exposure to IR is similar to that of the well-characterized, direct, p53 target gene *CDKN1A* ($p21^{Waf1}$). Despite the robustness of the p53 dependence of *PPM1D* induction in human and mouse cells and tissues, evidence for the direct control of this induction by p53 has been lacking.

Basal expression of *PPM1D* exhibits tissue and developmental stage-specific variation that normally is tightly regulated (1,2). Cells derived from $Ppm1d^{-/-}$ mice exhibit a slower doubling time, suggesting that Ppm1d also functions in the normal, regulated proliferation of cells (7). Overexpression of PPM1D is frequently observed in several types of cancer, including breast cancer (8), ovarian adenocarcinoma (9), and neuroblastoma (10) and the tumors in which PPM1D is overexpressed frequently contain wild-type p53. PPM1D functions in a negative regulatory loop coordinating p38 MAPK activity and p53 function (11). PPM1D negatively regulates stress response

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signaling by specifically dephosphorylating the protein kinase p38 MAPK (12). Since p53 is a target of p38 MAPK signaling, this activity also indirectly reduces p53 activity (13). More recently, Ser15 of p53 as well as Ser1981 of ATM were found to be directly dephosphorylated by PPM1D (14,15).

PPM1D was identified as a potential cyclic AMP response element (CRE) binding protein (CREB) target gene in a genome-wide comparison that examined the sequence and positional conservation of CRE sites in human and homologous mouse genes (16). CREB and the activating transcription factors (ATF) constitute a family of closely related proteins that bind to CRE (5′-TGACGTCA-3′) sequences present in the promoter regions of many genes involved in the response to proliferation signals (17). Subsequently, a genome-wide localization analysis detected highly significant association of CREB with chromatin in the region of the *PPM1D* promoter in human kidney, hepatocyte and islet cells (18). These results suggest that CREB contributes to the control of the basal expression of *PPM1D*.

p53 and CREB directly associate through protein–protein interactions involving the N-terminal activation domain of p53 and the bZip domain of CREB, implying that CREB and possibly other CREB/ATF family members may influence the transactivation of p53 target genes (19). Recently, a complex containing p53, CREB and KLF4 was found to control the basal expression of the bradykinin B2 receptor promoter in the developing kidney (20).

PPM1D induction after treatments that damage DNA occurs with similar kinetics in human and mouse cells and is similarly dependent on wild-type p53 (2,7). In the present work, we report that the p53-dependent induction of *PPM1D* expression in human cells after exposure to IR or UV is mediated through a conserved p53 response element (p53RE) located within the 5′ untranslated region (UTR), 150 nt before the translation initiation codon. In the absence of stress, the binding of CREB or a CREB/ATF family transcription factor contributes to the basal transcription of *PPM1D*. Increased expression of *PPM1D* following DNA-damaging treatments requires the presence of wild-type p53. Here we show that binding of p53 to the p53 RE within the proximal promoter shifts the major site of transcription initiation closer to the beginning of the PPM1D open reading frame, thereby producing mRNAs with shorter 5′ UTRs that may be more efficiently translated. Thus the location of the p53 RE within the 5′ UTR provides a mechanism for transcriptional and post-transcriptional regulation of the *PPM1D* gene by p53.

MATERIALS AND METHODS

Sequence analysis

Putative p53 binding sites were identified using the p53MH algorithm (21) with the following parameters: unweighted gap sizes from 0 to 13; core weighting factor, 2; likelihood ratio scoring method; minimum score, 21.5. Alignment of orthologous *PPM1D* genes and flanking regions used the multiz method (22). Phylogenetic analysis of aligned *PPM1D* proximal promoter regions used the program PHYLIP (23). The genomic locations for ten vertebrate sequences aligned to the human region [hg18 chr17: 56032232–56032681 (+)] are given in the Supplementary Data.

Cell culture and treatments

The HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cell lines (24), kind gifts from B. Vogelstein, Johns Hopkins University, USA, were propagated in McCoy's 5a or DMEM containing 10% fetal bovine serum (FBS) at 37°C in an atmosphere of 5% CO_2/air. Cells were exposed to the indicated doses of ionizing radiation from a ^{137}Cs Shepherd Mark II irradiator or of UV (254 nm, UVC) from a Stratalinker 1800 (Stratagene). Where indicated, cycloheximide (Sigma) was added to a final concentration of 10 μM.

Northern and western analyses

HCT116 $p53^{+/+}$ or HCT116 $p53^{-/-}$ cells were untreated or irradiated with 6 Gy IR and incubated 3 or 6 h. RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. The amount of purified RNA was determined spectrophotometrically. RNA samples were separated by electrophoresis in a formaldehyde-agarose gel, transferred to a Nytran membrane (Schleicher & Schuell) by capillary action and crosslinked by UV irradiation. *PPM1D* and *GAPDH* cDNA probes were labeled with ^{32}P-dCTP using a random primer labeling kit (Stratagene). After washing of the blot, hybridizing bands were visualized by autoradiography using Kodak AR-5 X-ray film with exposures of 1–5 days. Proteins from cell extracts were separated by gel electrophoresis on NuPAGE 7% Tris–Acetate Gels (Invitrogen) and transferred to nitrocellulose membranes (Invitrogen). Primary antibodies used: PPM1D, custom preparation by BD Biosciences; p53, DO-1 (Santa Cruz Biotechnologies); β-actin, AC15 (Sigma); α-tubulin, Ab-1 (Calbiochem); secondary antibody: HRP-conjugated antimouse IgG (Jackson Laboratories).

Plasmid construction

Reporter vectors were constructed by inserting the 849-bp EcoRI to BamHI, the 545-bp EarI to BamHI or the 454-bp Sau3AI to BamHI fragment containing the human *PPM1D* promoter sequence into the pGL3-basic vector (Promega). Mutations were introduced using the Quickchange Mutagenesis Kit II (Stratagene). The sequences of oligonucleotides used for mutagenesis are given in the Supplementary Data. In the construct *m*(123), central positions of the three p53 half-sites were changed to yield the following sequence, 5′-GGCaC AaCTCTCGCGGAtAAcTCCAGAtATCGCG-3′ where lower case letters indicate the sites of mutation. In constructs *m*(1) or *m*(3), only the first or third half-site was mutated. In the construct *mCRE*, the 8-base CRE was mutated to 5′-aGAgGTCt-3′. The construct *mm* contained all constituent mutations of the *m*(123) and *mCRE* constructs. All mutations were confirmed by sequencing. The plasmid pGL3/PUMA 0.9 kb, which contains a 0.9-kb fragment of the human *BBC3* promoter (PUMA) (25)

was a kind gift from G. Zambetti. The plasmid pGL3 E1bTATA-p21 (26) was a kind gift from S. Benchimol. Minimal promoter constructs containing the *PPM1D* p53RE and the analogous *m*(1), *m*(3) and *m*(123) variants were derived from pGL3 E1bTATA-p21 (26), as described in the Supplementary Data.

Transfection and luciferase assays

Transient transfections of HCT116 $p53^{-/-}$ cells were performed using Lipofectamine2000 (Invitrogen), 100 ng of pCAG3.1/p53 wild-type or pCAG3.1 (27), 200 ng of the Firefly luciferase reporter construct and 10 ng pRL-TK (Promega) per 3×10^5 cells. Twenty hours after transfection, cells were irradiated with 10 Gy from a ^{137}Cs Shepherd Mark II irradiator. After an additional 4 h of incubation, irradiated and nonirradiated cells were harvested and measured for *Photinus pyralis* (firefly) and *Renilla reniformis* (sea pansy) luciferase activities using the Dual Luciferase Assay System (Promega). In each experiment, *Renilla* luciferase activities from cells expressing wild-type p53 were corrected for the ~1.9-fold increase in activity of pRL-TK in the presence of wild-type p53 by the ratio given by: mean activity (with p53)/mean activity (without p53). Well-to-well variation was on the order of 10%. To correct for differences in transfection efficiency, the reported relative luciferase activity of each sample is taken as the ratio of the Firefly luciferase activity to the corrected *Renilla* luciferase activity. Results represent the average ± standard deviations of two or three independent experiments.

Chromatin immunoprecipitation assay

Subconfluent HCT116 $p53^{+/+}$ or HCT116 $p53^{-/-}$ cells were exposed to 10 Gy IR or left untreated, using two dishes of 3×10^6 cells per time point. Chromatin immunoprecipitation (ChIP) experiments were performed using a kit (Upstate) and following a modified version of the manufacturer's protocol. A detailed protocol is given in the Supplementary Data. The following antibodies were used: p53, DO-7 (Calbiochem OP140 or Neomarkers); CREB (Upstate 06-863); acetyl histone H3 (Upstate 06-599), acetyl-histone H4 (Upstate 06-866). The presence of *PPM1D* promoter or distal p21 promoter fragments in the immunoprecipitated material was detected by PCR; details are given in the Supplementary Data.

Analysis of transcription initiation site usage by PCR

Total RNA was purified from treated or untreated HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells, with inclusion of treatment with DNaseI to remove genomic DNA. Reverse transcription was performed with SuperScript reverse transcriptase II (Invitrogen) and an oligo dT primer. Primers for the detection of *PPM1D* transcripts were designed using Light Cycler Probe Design Software 2.0 (Roche). Real-time PCR (RT-PCR) was performed with Light Cycler FastStart DNA Master SYBR Green I (Roche) and a LightCycler Real-Time PCR instrument (Roche) using a common reverse primer 5′-TGGGC CTTTCCCCGAGA-3′ located in exon 1 of *PPM1D* (+204 bases from the ATG) and either of two forward primers: primer 1 (5′-GGCGTCGTCGAAGATAAAC AATA-3′, 106 bases upstream of the ATG) or primer 2 (5′-GGACGTTACTCAAATCGTTGT-3′, +66 bases from the ATG). RT-PCR was performed in triplicate on each sample, using a final concentration of magnesium chloride of 3 mM. RT-PCR curves were analyzed using PCR Miner software (28) to determine the efficiency for each primer pair and the initial transcript abundances for each sample. Transcript abundances for *PPM1D* transcripts were normalized to β-actin (29) transcript levels. The standard deviations of the means were estimated by propagation of error calculations.

RESULTS

p53-dependent induction of *PPM1D* mRNA and protein following exposure to IR

To investigate the mechanism of the p53-dependent induction of *PPM1D*, we used the human colorectal cell line HCT116, which contains wild-type p53 protein and exhibits a normal p53 response following treatments with DNA damage-inducing agents, and an HCT116 $p53^{-/-}$ derivative that lacks p53. Northern blot analysis of *PPM1D* expression in HCT116 $p53^{+/+}$ cells showed that *PPM1D* mRNA levels increased after exposure to 10 Gy IR in $p53^{+/+}$ cells with the maximum induction occurring 3 h post-irradiation (Figure 1A). Although the levels of *PPM1D* mRNA were comparable in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells prior to irradiation, *PPM1D* mRNA levels decreased after IR in $p53^{-/-}$ cells. As shown in the immunoblot depicted in Figure 1B, the levels of PPM1D protein also increase markedly following exposure of cells containing wild-type p53 to IR. These results are in accord

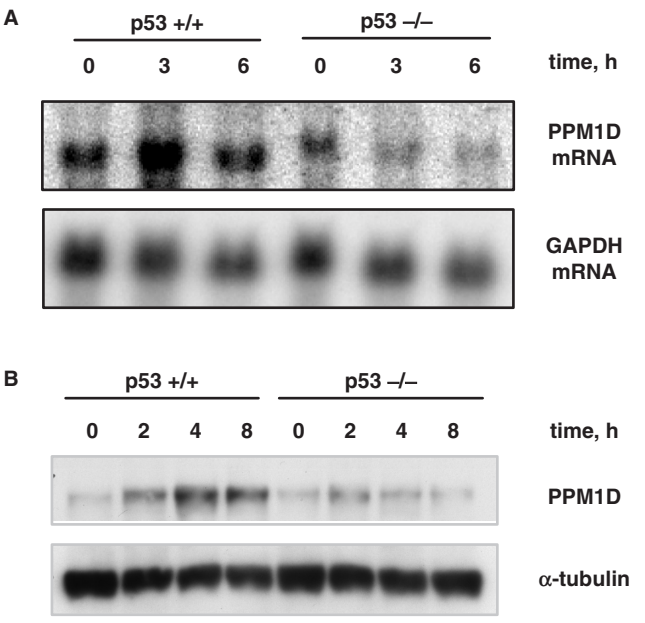

Figure 1. (**A**) Northern blot analysis of *PPM1D* expression in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells following exposure to 6-Gy IR. (**B**) Immunoblot analysis of PPM1D protein levels in extracts from HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells at the indicated times following exposure to 10-Gy IR.

with previous work establishing that the induction of *PPM1D* mRNA and protein following IR in human or mouse cells is dependent on wild-type p53 (1,2).

Identification of conserved p53 consensus sequences in the human *PPM1D* and mouse *Ppm1d* promoters

Genes shown to be directly induced by p53 generally contain a p53RE in the promoter or within the first few introns. A pattern that has been used to identify potential p53REs consists of two repeats of the pattern RRRCWWGYYY separated by a spacer of 0–13 bp (30), although p53 is known to bind to additional patterns (31,32). A recent global analysis of sequences bound by p53 suggested that spacers other than 0 or 1 are rare (33). To locate potential p53REs regulating *PPM1D* expression, we applied the p53 MH algorithm (21) to the human *PPM1D* and mouse *Ppm1d* genomic sequences from 5 kb upstream of the translation start site through the 3′ UTR. Using a cutoff of 21.5 but allowing non-zero spacers, we identified eight potential p53REs in the human sequence and 18 in the mouse sequence. For both species, two of these high-scoring sites are located in the proximal promoter. Interestingly, both sites reside in regions of high sequence conservation among mammals (34). The sequences of these potential p53REs and their location relative to the respective translation initiation site are given in Figure 2A. For the human gene, the upstream site matches the consensus pattern at 17 of 20 positions. The upstream site in the mouse gene is located at a similar distance from the translation initiation site but does not align exactly with the human p53 RE site. The downstream p53RE identified by the p53 MH algorithm consists of two half-sites separated by a spacer of 4 bp that match the consensus at 18 of 20 positions. The human and mouse sequences are identical over this 24-bp span. A third half-site, which deviates from the consensus pattern at three positions, immediately follows the second half-site. Interestingly, the combination of the second and third half-sites was identified as a conserved p53-binding site through application of a position-weight matrix method to aligned human, mouse and rat genomic sequences (35).

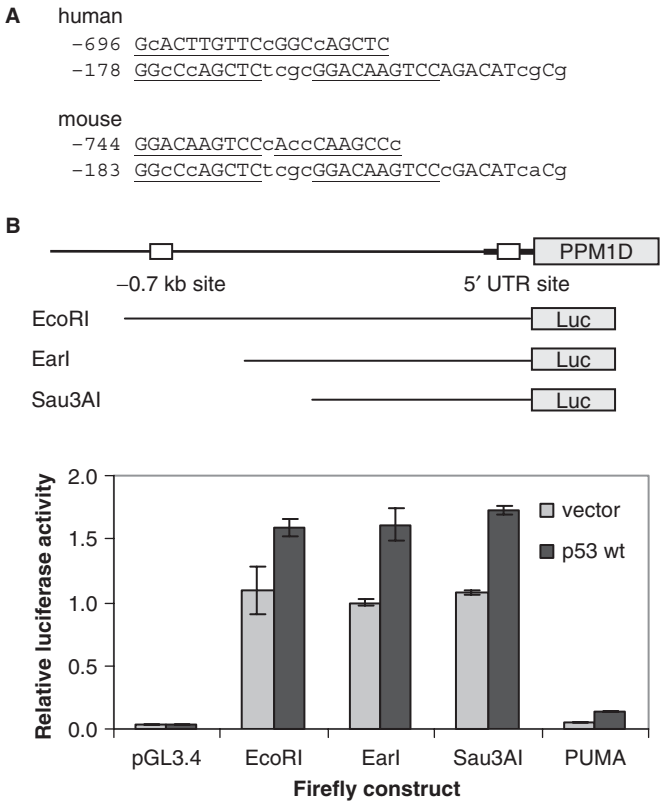

Figure 2. (**A**) Sequences of potential p53REs in the human *PPM1D* and mouse *Ppm1d* promoters. Distances in base pairs from the translation initiation codon are given. Bases that match the p53 consensus pattern are written in upper case and decameric half-sites identified by the P53MH algorithm are underlined. (**B**) Truncation analysis of the *PPM1D* promoter using reporter constructs. A schematic of the *PPM1D* promoter region and derived luciferase reporter constructs is shown. The relative locations of the –0.7-kb and 5′ UTR potential p53 response elements are indicated. The relative firefly luciferase activities in extracts of HCT116 $p53^{-/-}$ cells transiently transfected with a reporter construct, pRL-TK and a wild-type p53 expression vector or empty vector are shown. Cellular extracts were prepared 4 h after exposure to 10-Gy IR. The *PUMA* construct served as a positive control. The means and standard deviations of three independent transfections are indicated.

Localization of p53-dependent induction to the *PPM1D* promoter region

To locate the region of the *PPM1D* promoter that is responsible for p53-dependent expression following exposure to IR, we cloned the 849-bp EcoRI to BamHI fragment containing the proximal promoter region into the pGL3-Luc vector upstream of the firefly luciferase gene. Two additional constructs containing 5′ truncations that deleted the upstream site were prepared in the same vector. A schematic of the promoter region of *PPM1D* and the derived reporter constructs is shown in Figure 2B with the relative location of the two putative p53 response elements indicated. Since most methods for transiently introducing reporter vector DNA into mammalian cells also result in the activation of p53 (36,37), we investigated the p53-dependence by co-transfecting a wild-type p53 expression vector (pCAG3.1/p53 wt) or the empty expression vector (pCAG3.1) (27) along with the pGL3-Luc derivative and pRL-TK (for normalization of transfection efficiency) into HCT116 $p53^{-/-}$ cells. The amount of the p53 expression vector was adjusted to produce a level of p53 similar to that produced in the related HCT116 $p53^{+/+}$ cells after exposure to 10 Gy IR. Cellular extracts were prepared 4 h after exposure to IR, and the resulting relative, normalized firefly luciferase activities are depicted in Figure 2B. The promoterless construct pGL3.4 produced a low level of activity either in the absence or presence of wild-type p53. Insertion of the 848-bp EcoRI to BamHI fragment of the *PPM1D* promoter into the pGL3-Luc vector resulted in substantial expression of firefly luciferase, even in the absence of p53, consistent with basal expression of *PPM1D* in most cells and tissues (1). The same construct resulted in 1.5-fold higher expression in the presence of wild-type p53. Constructs containing

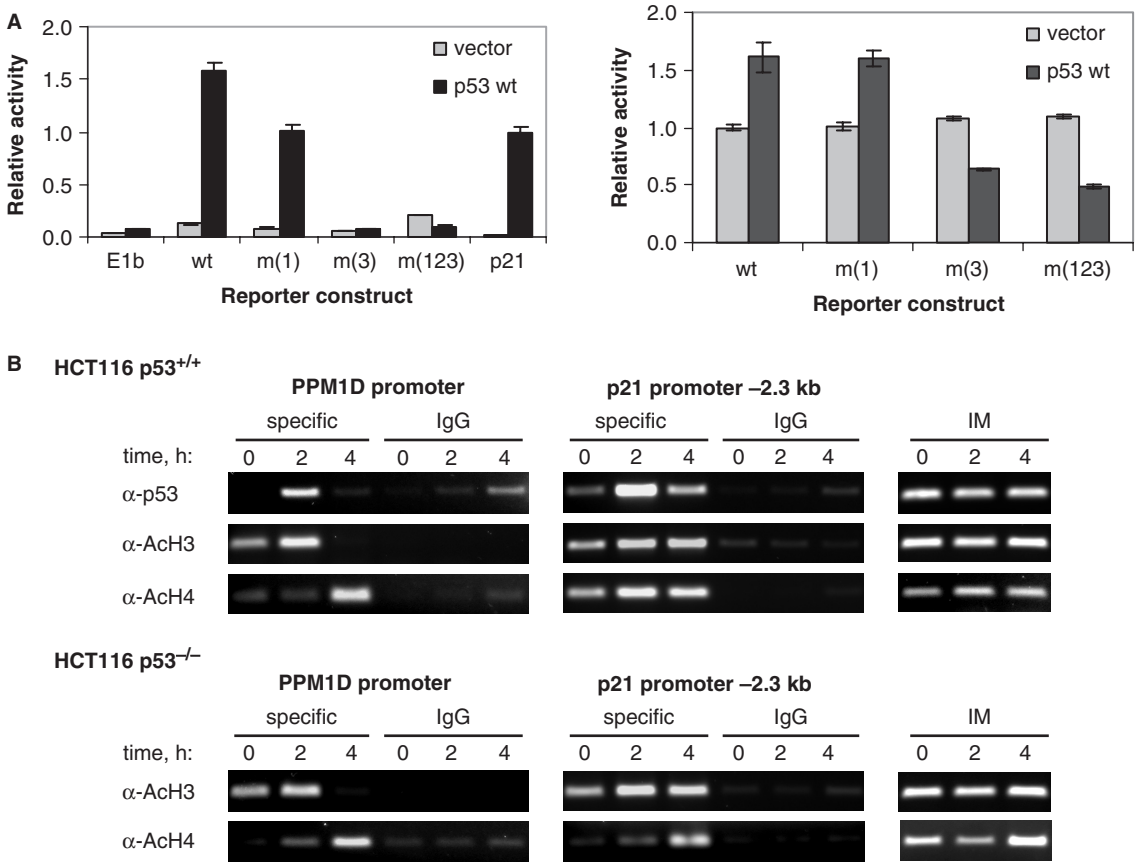

Figure 3. Functional analysis of p53 binding to *PPM1D* proximal promoter sequences. (**A**) Functional analysis of the *PPM1D* p53RE. HCT116 $p53^{-/-}$ cells were transfected with E1b minimal promoter reporter constructs (left panel) or *PPM1D* proximal promoter reporter constructs (right panel), along with pRL-TK and an empty vector or a wild-type p53 expression vector. Cellular extracts were prepared 4 h after exposure to 10-Gy IR. The means and standard deviations of three independent transfections are indicated. (**B**) Association of p53 or acetylated histones with *PPM1D* or p21 chromatin fragments following exposure to IR. HCT116 $p53^{+/+}$ or $p53^{-/-}$ cells were untreated or exposed to 10-Gy IR and samples were harvested after the indicated times. Chromatin fragments immunoprecipitated by the anti-p53 antibody DO-7, anti-acetyl histone H3, anti-acetyl histone H4 or nonspecific IgG were detected by PCR using primers specific for the *PPM1D* promoter or the distal (–2.3 kb) p21 promoter region. Fragments amplified from 0.5% of the input material are shown.

shorter *PPM1D* promoter fragments similarly resulted in substantial luciferase activity in the absence of p53 and ~1.6-fold higher levels in the presence of wild-type p53. The pGL3 derivative containing the promoter region of the human *BBC3* (PUMA) gene produced only a low level of luciferase activity in the absence of p53, but the activity increased ~2.6-fold in the presence of p53, as expected (25). These experiments show that the *PPM1D* promoter region exhibits substantial p53-independent transcription initiation activity in HCT116 cells and that the increased expression in the presence of wild-type p53 results from components within the 470 bp upstream of the translation start site. Under our conditions, the –0.7-kb site does not contribute to the observed p53-dependent increased activity of the *PPM1D* promoter. However, the presence of a nearby conserved E2F-binding site (38) in the human sequence and the identification of a potential p53 RE in the mouse *Ppm1d* promoter at similar distances from the respective E2F site and translation initiation codon suggests that the upstream p53-binding sites may be functional in some circumstances.

The p53RE located within the 5′ UTR conferred p53-responsiveness to the *PPM1D* promoter

The putative p53-binding site is situated downstream of the transcription initiation site of the *PPM1D* reference mRNA (NM_003620) and is located 178 bp upstream of the translation start site. The ability of the *PPM1D* p53RE site to confer a p53-dependent increase in transcription to a minimal promoter was tested by transient transfection of HCT116 $p53^{-/-}$ cells with firefly luciferase constructs, pRL-TK and a p53 expression vector or the empty vector. As shown in Figure 3A, left panel, pGL3 derivatives containing the viral oncoprotein E1b minimal promoter exhibited little promoter activity either in the absence or presence of wild-type p53 (26). Insertion of the p53 response element from the p21 promoter (26) resulted in a low level of expression in the absence of p53 that was strongly increased in the presence of p53. Insertion of the 36-bp sequence encompassing the *PPM1D* p53RE site or any of the mutated forms into the pGL3/E1b TATA vector resulted in only slightly increased expression in the absence of p53. Luciferase

activity resulting from expression from the construct containing the *PPM1D* p53RE sequence was strongly increased in the presence of wild-type p53, exhibiting about a 12-fold increase over that in the absence of p53. In comparison with the wild-type p53RE sequence, mutation of the first half-site reduced the expression level in the presence of p53 by 35%. Mutation of the third half-site or all three half-sites abolished p53-dependent expression.

To test the functioning of the *PPM1D* p53RE within the context of the native *PPM1D* promoter, we introduced the same series of mutations into the pGL3-Luc derivative containing the EarI to BamHI fragment of the *PPM1D* promoter region and examined the effect of p53 expression on *PPM1D* promoter activity in the transient transfection assay. In the absence of p53, the wild-type promoter and the three mutant forms resulted in very similar levels of luciferase activity (Figure 3A, right panel). In the presence of p53, the wild-type promoter and the *m*(1) mutant each resulted in ~1.6-fold higher expression, while the *m*(3) and *m*(123) mutants resulted in activities that were 64% and 49%, respectively, that of the wild-type promoter. The lack of a measurable effect of mutation of the first half-site within p53RE suggests that the second and third half sites form the functional p53 response element under these conditions. The finding that the *m*(3) and *m*(123) mutants result in significantly lower luciferase activity in the presence of p53 than in its absence is interesting and may result from a general repressive effect of p53 on transcription (39,40) or from the p53-dependent induction of a repressor. The more robust p53-dependence exhibited by the minimal promoter constructs suggests that the observed p53-responsiveness of the intact promoter may reflect the balance of positive and negative contributions.

To determine whether p53 bound to the *PPM1D* promoter region *in vivo*, we performed ChIP experiments. As shown in Figure 3B, chromatin fragments containing the *PPM1D* promoter region were recovered by the anti-p53 antibody DO-7 from HCT116 $p53^{+/+}$ cells 2 h after exposure to IR, but the amount recovered fell to background levels by 4 h after exposure. The transient presence of p53 in the *PPM1D* promoter region corresponds to the transient increase in *PPM1D* mRNA (1). We also tested for p53 association with the –0.7-kb site, but recovered only background levels of chromatin fragments (data not shown). The amounts of p21 upstream promoter fragments that were recovered from material immunoprecipitated by the anti-p53 antibody followed the expected pattern (41). Furthermore, we examined changes in histone acetylation patterns in the *PPM1D* and p21 promoter regions following exposure to IR. Acetylation of histones H3 and H4 in *PPM1D* promoter fragments exhibited distinct changes following exposure to IR, but the pattern was very similar in HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells. In both cell lines, histone H3 acetylation was detectable in untreated samples, increased by 2 h after exposure to IR and fell to background levels by 4 h after exposure to IR. Histone H4 acetylation was not detected in the untreated or 2-h samples but was apparent 4 h following exposure to IR (Figure 3B). In contrast, although histone H3 acetylation increased in p21 upstream promoter fragments following exposure to IR in both HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells, the strong increase in histone H4 acetylation 2 h after exposure to IR was observed only in cells containing wild-type p53, as reported previously for the p21 and *BBC3* (PUMA) promoters (42,43). These results suggest that modulation of histone acetylation is less important in the p53-dependent activation of the *PPM1D* promoter than for the p21 promoter. Transcription of the p21 gene has been shown to be exceptionally sensitive to manipulation of histone acetylation levels (44).

Phylogenetic conservation of the p53RE and the *PPM1D* core promoter

The amino acid sequence of PPM1D is sufficiently well conserved that its emergence as a distinct member of the PP2C superfamily can be inferred to have occurred prior to the divergence of the protostomes (45). Promoter and regulatory elements often diverge more rapidly than protein sequences. A schematic diagram (Figure 4A) depicts features of the *PPM1D* core promoter aligned with a graph indicating the degree of evolutionary conservation among 17 vertebrate species (34). Much of the core promoter, including the p53RE and the CRE, is highly conserved. To further investigate sequence conservation in the *PPM1D* core promoter, we applied a phylogenetic inference algorithm to a 450-bp sequence of the human *PPM1D* promoter and the aligned sequences of 10 other species (23). An unrooted tree diagram indicating the relatedness of *PPM1D* core promoter sequences is shown in Figure 4B. The aligned sequence from the chicken genome is markedly divergent except for the included portion of exon 1, which is well conserved. Thus the *PPM1D* core promoter sequence is highly conserved among mammals, especially placental mammals. An alignment of the sequences encompassing the *PPM1D* p53RE for 10 mammalian species (Figure 4C) indicates that all three half-sites are highly conserved among placental mammals. Although the opossum sequence is the most divergent, the middle half-site matches the p53 consensus pattern perfectly.

The CRE site is not necessary for the p53-dependent induction of *PPM1D* after IR

The presence of a conserved CRE within the promoter of the *PPM1D* gene (16), reported protein–protein interactions between p53 and CREB (19) and reported cooperation in the control of the Bradykinin B2 receptor gene by p53 and CREB (20) suggested a possible involvement of the CRE site and CREB/ATF family transcription factors in the IR-responsiveness of the *PPM1D* promoter. To investigate whether the CRE site was necessary for the p53-dependent induction of the *PPM1D* promoter, we transiently transfected expression vectors and luciferase reporter constructs into HCT116 $p53^{-/-}$ cells. In agreement with results described above, the relative luciferase activity resulting from the wild-type *PPM1D* promoter was robust in the absence of p53 and increased ~1.5-fold in the presence of p53; mutation of p53RE [*m*(123)] did not affect the promoter activity in the absence

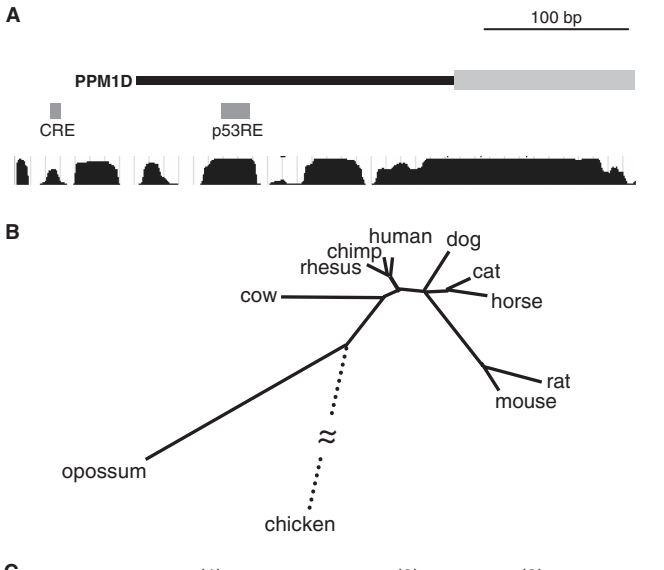

Figure 4. Sequence conservation of the *PPM1D* core promoter and p53RE. (**A**) Schematic diagram depicting the genomic region around the *PPM1D* 5′ UTR. The relative positions of the *PPM1D* reference mRNA and protein are indicated by thin and thick gray lines, respectively, the positions of the CRE and p53RE are indicated by rectangles and the lower plot displays the conservation score based on an alignment of 17 vertebrate species. The diagram is based on the UCSC Genome Browser display (35). (**B**) Unrooted phylogenetic tree of the *PPM1D* core promoter for 10 mammalian species. The 450-bp sequence of the human *PPM1D* core promoter, 5′ UTR and portion of exon 1 and aligned sequences from nine additional mammalian species, plus the chicken sequence, were analyzed by the PHYLIP program (23). (**C**) Sequence conservation of the *PPM1D* 5′ UTR p53RE. Alignment of a 38-bp region from 10 mammalian species is shown. Numbered gray bars indicate the three half sites. Positions that conform to the consensus are indicated by upper case letters. The human sequence is written in bold.

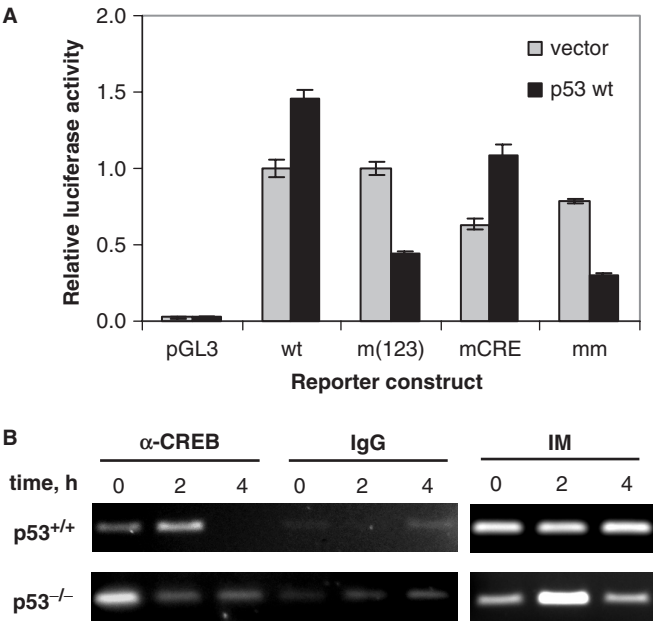

Figure 5. Involvement of the CRE and CREB in the control of *PPM1D* transcription following exposure to IR. (**A**) The CRE and p53RE sites independently regulate *PPM1D* promoter activity. HCT116 $p53^{-/-}$ cells were transfected with constructs containing a 600-bp *PPM1D* promoter fragment or with mutations in the p53RE site, the CRE site or both sites, along with pRL-TK and the empty vector or wild-type p53 expression vector. Cellular extracts were prepared 4 h after exposure to 10 Gy IR. (**B**) CREB dissociates from the *PPM1D* promoter region following exposure to IR. Chromatin fragments immunoprecipitated by anti-CREB antibody or nonspecific IgG from HCT116 $p53^{+/+}$ or HCT116 $p53^{-/-}$ cell extracts at the indicated times following exposure to 5-Gy IR were detected by PCR using primers specific for the *PPM1D* promoter. Fragments amplified by PCR from 0.62% of the input material are shown.

of p53 but reduced promoter activity in the presence of p53 to 44% (Figure 5A). In the absence of p53, mutation of the CRE reduced the promoter activity to 63% of the wild-type promoter, but in the presence of p53, the CRE-mutated promoter exhibited 1.7-fold increased expression. The construct in which both the CRE and p53RE sites (*mm*) were mutated produced an intermediate level of expression in the absence of p53 and a reduced expression in the presence of p53.

Since the process of transfection results in partially activated p53, all reporter experiments were performed with extracts from irradiated cells in order to fully activate p53. These experiments thus provide information about promoter activity after irradiation in the absence or presence of activated wild-type p53. The data are consistent with a model in which transcription factors binding to the CRE and p53RE sites independently affect the level of transcription after exposure to IR. In either the absence or presence of p53 and with either the wild-type promoter or the p53RE mutated promoter, mutation of the CRE reduced the promoter activity to ~70% of the activity of the corresponding construct with the intact CRE (Figure 5A). Mutation of the p53RE had little effect on the promoter activity in the absence of p53, but in the presence of p53, mutation of the p53RE reduced the promoter activity to ~30% that of the wild-type p53RE promoter. Consistent with sequence-specific, DNA-binding-dependent transcriptional activation by p53, constructs with an intact p53RE exhibited increased expression in the presence of p53, with 1.5- and 1.7-fold increases observed for the wild-type and *m*CRE constructs, respectively. Conversely, constructs with a mutated p53RE exhibit a reduced activity in the presence of p53, with relative activities of 0.44 and 0.38 observed for the *m*(123) and *mm* constructs, respectively, compared to the activity in the absence of p53. The reduced activity of *PPM1D* promoter constructs that lack an intact p53RE may be the result of a p53-dependent induction of a transcriptional repressor that functions on the *PPM1D*

promoter or may result from a generalized repressive effect of p53 on transcription from TATA-less promoters (40). Thus, in the context of a transiently transfected reporter plasmid, we observed no obvious co-operativity between CRE-based and p53RE-based transcriptional activation.

Ionizing radiation-induced changes in CREB binding to the *PPM1D* promoter in chromatin

To investigate the binding of CREB to the *PPM1D* promoter in native chromatin, we performed ChIP experiments. As shown in Figure 5B, *PPM1D* promoter fragments were associated with CREB in unirradiated HCT116 $p53^{+/+}$ cells, and the amount increased at 2 h after exposure to IR before declining to background levels by 4 h after exposure to IR. In HCT116 $p53^{-/-}$ cells, the association of *PPM1D* promoter fragments with CREB was detected in untreated samples while only background levels were recovered from samples taken 2 or 4 h after exposure to IR. The presence of detectable CREB binding to the *PPM1D* promoter region at 2 h after exposure to IR in HCT116 $p53^{+/+}$ but not in HCT116 $p53^{-/-}$ cells may reflect a direct interaction between p53 and CREB in the context of the chromatin of the *PPM1D* promoter or it may result from an indirect effect of the p53-dependent response to IR. Since the total amount of CREB in HCT116 $p53^{+/+}$ or HCT116 $p53^{-/-}$ cells increased only moderately following exposure to IR (data not shown) and the affinity of CREB/ATF family transcription factors for their cognate recognition sequence is not thought to be affected by their phosphorylation status (46), the observed absence of CREB associated with the *PPM1D* promoter at 4 h following exposure to IR may be due to its displacement by a factor with higher affinity for the CRE.

p53-dependent shift in the use of transcription initiation sites following IR

The *PPM1D* promoter is GC-rich, TATA-less and lacks an initiator element. Some promoters with these characteristics exhibit a distributed pattern of transcription start sites (TSS) (47). Based on a database of TSS (48), the 38 *PPM1D* mRNAs initiated at 14 different positions, which formed two clusters, 230 to 200 bp and 95 to 65 bp before the ATG. The two most prevalent sites, which produce transcripts with 222 or 65 base 5′ UTRs, account for 60% of the transcripts. The CRE is located 60 bp before the most upstream of the mRNA start sites, within the typical range for functional CREs (46). The p53RE is located about 60 bp upstream of the second cluster of TSS. A schematic diagram, depicting the proximal promoter of *PPM1D*, the CRE and p53RE sites, and the two most prevalent transcripts, is shown in Figure 6A.

We used a quantitative PCR-based method (28) to examine whether p53 affected the pattern of transcriptional initiation of the PPM1D gene in HCT116 cells, either in untreated cells or in cells exposed to10 Gy IR. One primer pair detects only transcripts initiating within the first cluster of initiation sites, whereas the second primer pair detects all transcripts. As shown in Figure 6B,

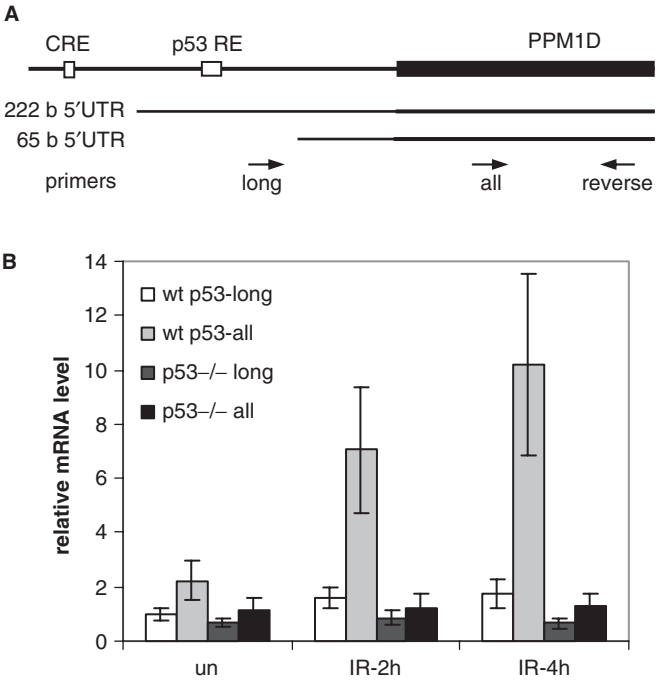

Figure 6. Patterns in the transcription start sites in the *PPM1D* promoter. (**A**) Schematic of transcription start sites for *PPM1D*. The top line depicts genomic DNA in the vicinity of the *PPM1D* 5′ UTR. The second and third lines represent the most prevalent transcripts in the two clusters of transcription start sites. The locations of the two forward and common reverse primers used for real-time PCR are indicated (**B**) p53-dependent shift in the pattern of transcription initiation in the *PPM1D* promoter in HCT116 $p53^{+/+}$or $p53^{-/-}$cells following exposure to 10 Gy IR. A quantitative PCR-based method was used to determine the relative abundances of *PPM1D* mRNAs with long 5′ UTRs compared to all *PPM1D* transcripts.

the abundance of long transcripts was not affected by the presence of wild-type p53 or by IR treatment. In contrast, the total abundance of *PPM1D* transcripts greatly increased in the HCT116 cells containing wild-type p53 after exposure to IR. These results demonstrate that the p53-dependent increase in transcription that occurs after exposure to IR results from transcripts that initiate downstream of the p53RE site. Additional evidence in support of these observations may be found in a genome-wide study of transcripts using a paired-end tagged sequencing method (49). Among the thousands of transcripts mapped in their study, 47 out of 59 *PPM1D* transcripts from exponentially growing MCF7 cells originated upstream of the p53RE site in the *PPM1D* promoter whereas in HCT116 $p53^{+/+}$ cells following treatment with 5-fluorouracil, 11 out of 16 *PPM1D* transcripts originated downstream of the p53RE site.

p53-dependent shift in the use of transcription initiation sites following UV is associated with increased PPM1D protein levels

The results described above demonstrated that the total amount of *PPM1D* mRNA, the fraction of *PPM1D* transcripts with short 5′ UTRs and PPM1D protein levels all increase following exposure to IR in cells with

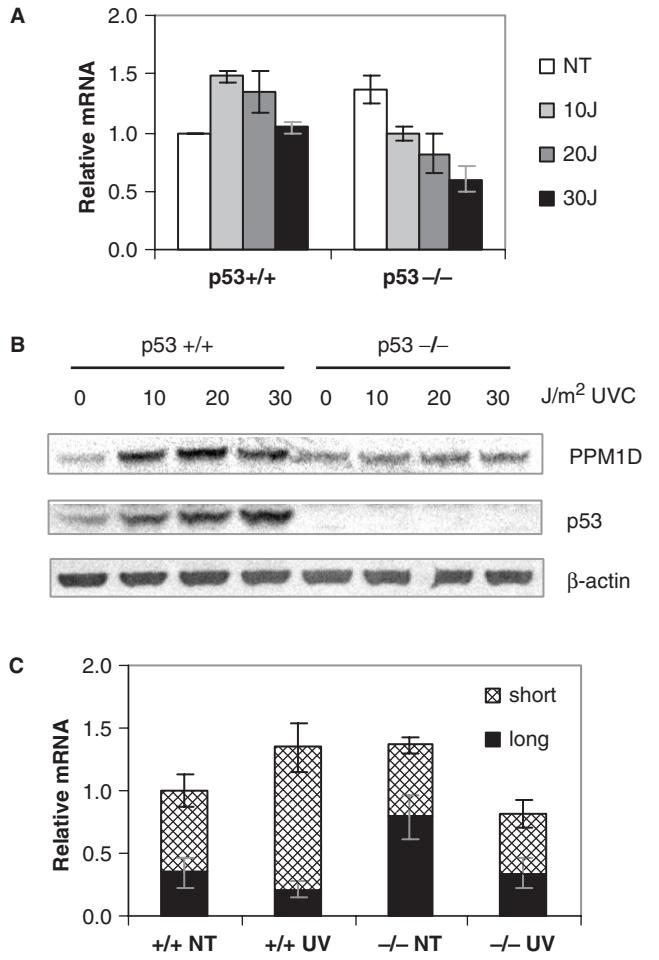

Figure 7. Effects of UV exposure on the *PPM1D* gene expression. (**A**) Effects of UV exposure on *PPM1D* mRNA levels. HCT116 $p53^{+/+}$ or $p53^{-/-}$ cells were untreated (NT) or exposed to the indicated doses of UV and total RNA was extracted 4 h later. The relative levels of *PPM1D* mRNAs were determined by quantitative RT-PCR, normalized to β-actin mRNA levels and expressed as a ratio of the level in untreated $p53^{+/+}$ cells. (**B**) Effects of UV exposure on *PPM1D* and p53 protein levels. HCT116 $p53^{+/+}$ or $p53^{-/-}$ cells were untreated (NT) or exposed to the indicated doses of UV, cellular proteins were extracted 6 h later and specific proteins were detected by immunoblot. (**C**) Patterns of transcription initiation in the *PPM1D* promoter following exposure to UV. Total RNA was isolated from untreated cells or 4 h after exposure to 20 J/m^2 UV. A quantitative PCR-based method was used to determine the relative abundances of *PPM1D* mRNAs with long 5′ UTRs compared to all *PPM1D* transcripts. The amount of *PPM1D* transcripts with short 5′ UTRs was determined by difference.

wild-type p53. To discriminate between the effects of the total *PPM1D* mRNA level and the abundance of transcripts with short 5′ UTRs, we examined the effects of exposure to UV on *PPM1D* mRNA and protein levels. The increased expression of *PPM1D* mRNA has been shown to be dependent on both p53 and p38 MAPK (12). However, due to the formation of transcription-blocking photoproducts within the 60-kb *PPM1D* gene, the production of full-length transcripts is constrained at higher levels of UV (50). As shown in Figure 7A, *PPM1D* mRNA levels increased in HCT116 $p53^{+/+}$ cells following exposure to 10 or 20 J/m^2 UV but was essentially unchanged following exposure to 30 J/m^2. The *PPM1D* mRNA level was slightly higher in untreated HT116 $p53^{-/-}$ cells than in $p53^{+/+}$ cells, as observed above (Figure 5B), and decreased with increasing UV dose. This pattern is consistent with a p53-dependent increase in transcription of the PPM1D gene following exposure to UV combined with a UV dose-dependent inhibition of production of full length transcripts (50).

PPM1D protein levels increased markedly following exposure to 10, 20 or 30 J/m^2 UV in $p53^{+/+}$ cells, with the greatest increase in cells exposed to 20 J/m^2 UV as shown in Figure 7B. Most importantly, PPM1D protein levels increased following exposure to 30 J/m^2, although the *PPM1D* mRNA level was the same as in untreated cells. In $p53^{-/-}$ cells, PPM1D protein levels in UV-treated cells exhibited a small increase compared to the level in untreated cells. We also observed that p53 protein levels exhibited a dose-dependent increase in HCT116 $p53^{+/+}$ cells following exposure to UV, as expected. The observed increase in PPM1D protein levels following exposure to UV may be due to an increase in the rate of protein synthesis, an increase in protein stability or a combination of the two effects. To determine the effects of UV exposure on PPM1D stability, we examined PPM1D protein levels following addition of the protein synthesis inhibitor cycloheximide by immunoblot (data not shown). PPM1D protein levels remained approximately constant in untreated HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells for up to 6 h following addition of cycloheximide. In contrast, in both $p53^{+/+}$ and $p53^{-/-}$cells that had been exposed to 30 J/m^2 UV 6 h previously, PPM1D protein levels decreased with an apparent half-life of about 3 h following addition of cycloheximide. These results suggest that PPM1D exhibits higher turnover following exposure to UV and, consequently, that the increased protein levels observed in $p53^{+/+}$ cells result from a combination of increased *PPM1D* mRNA levels and an increased utilization of the mRNA for protein synthesis.

We used the same PCR-based method to examine UV-induced changes in the utilization of transcription start sites in the *PPM1D* promoter. As shown in Figure 7C, the increased transcription of the *PPM1D* gene that occurred following exposure to UV in HCT116 $p53^{+/+}$ cells resulted from increased production of transcripts with short 5′ UTRs and decreased production of transcripts with long 5′ UTRs. In $p53^{-/-}$ cells, however, exposure to UV resulted only in the decreased production of transcripts with a long 5′ UTR with no change in the number of transcripts with a short 5′ UTR, leading to a decrease in the total level of *PPM1D* transcripts. Thus the increase in PPM1D protein levels observed in $p53^{+/+}$ cells correlated with the increase in production of transcripts with short 5′UTRs.

DISCUSSION

Implications of p53REs located near transcription initiation sites

In response to DNA damage and other stress signaling, wild-type p53 positively or negatively affects the

transcription of a large number of genes, possibly hundreds (6,51). Typically, transcriptional activation of a particular gene by p53 involves the stabilization and activation of p53 by post-translational modifications (52), the binding of p53 to a response element located usually either within 1 kb upstream of the promoter or within the first few introns (21), the recruitment of transcriptional co-activators that acetylate the tails of nearby histones (42,53) that result in increased transcription. Several distinct mechanisms have been found to operate in the p53-dependent repression of specific genes, including recruitment of histone deacetylases (54,55) and displacement of essential activating transcription factors (56,57). The phosphatase PPM1D has been shown to be induced after exposure to ionizing radiation with the rapid kinetics characteristic of primary targets of p53 (1), but the mechanism was not known. In the present work, we identified a functional p53RE in the proximal promoter region of the *PPM1D* gene that lies within the 5′ UTR. Further experiments showed that after exposure to IR or UV, transcription initiation increased at sites closer to the initiation codon in cells with wild-type p53. Following exposure to UV, the increased abundance of transcripts with shorter 5′ UTRs correlated with an increased rate of PPM1D synthesis. The length of the 5′ UTR may affect the rate of nuclear export of the mRNA or the rate of initiation of protein synthesis (58,59). Thus, the location of the p53 RE within the 5′ UTR and the resulting shift in transcription initiation site usage provides a mechanism for post-transcriptional as well as transcriptional regulation of *PPM1D* expression by p53.

Although the p53-binding site identified by the p53 MH algorithm contained a spacer of 4 bp, our analysis (Figure 3) indicated that the functional site consists of the downstream decameric half-site plus the adjacent 10-bp sequence. This site matches the consensus sequence at 10/10 and 7/10 positions, with no spacer between the two decamers. Recent work suggests that most functional p53-binding sites have no spacer between the decameric half-sites (33). A survey of functional p53 REs (60,61), revealed that many sites deviate substantially from the consensus sequence. Moreover, of the two p53 REs in the p21 promoter region, the distal site has a better match to the consensus sequence and higher binding of p53, but it is the occupancy of the proximal site that correlates with increased histone acetylation and increased transcription (42).

The location of a p53-binding site within the 5′ UTR is unusual but not unprecedented. Two other well-characterized p53-responsive genes contain p53REs within their 5′ UTRs. The gene for LRDD, also known as PIDD, is a pro-apoptotic gene induced by p53 following DNA damage, and it contains a conserved p53RE located 26 nt before the translational initiation codon (62). In contrast, *DNMT1*, which encodes a CpG-specific methyltransferase, is repressed by wild-type p53 in the absence of DNA damage, but after treatment with DNA damage-inducing agents, the amount of bound p53 decreases and transcription of *DNMT1* increases (63). The identified p53RE is located 129 nt upstream of the translational initiation codon ATG but after the point of transcription initiation, a location that can be rationalized with repression of *DNMT1* transcription by bound p53. For *PPM1D*, the p53-dependent shift in the site of transcription initiation after IR means that the p53RE is close to but still upstream from the TSS, consistent with the activation of *PPM1D* transcription by p53. Furthermore, although the recruitment of histone acetyltransferases is generally considered to be the main mechanism through which p53 activates transcription of target genes (64), p53 has been shown to interact with other components of the transcription machinery (65–67). The direct interaction of p53 with transcription complexes may be more important in the transcriptional activation of genes that have a functional p53 response element located near the transcriptional start site.

Although the function of p53 as a modulator of transcription is, perhaps, the best understood of its many activities, the regulation of transcription and of translation are interrelated. The expression of p53, itself, is regulated at many levels, including translation (68) and an alteration in the transcriptional start sites of p53 was found to occur in tumor cells (69). The p53-dependent production of alternate transcripts of the p21 gene has been reported recently (70). Interestingly, a p53-dependent change in promoter usage was shown to produce a 5′ UTR with a different sequence for the human MDM2 mRNA and, by consequence, change the AUG used for the initiation of protein synthesis (71,72). In the case of *PPM1D*, the location of a p53-binding site so close to the translational start codon suggests that the level of PPM1D protein also may be subject to post-transcriptional regulation. The increased utilization of *PPM1D* transcripts in the production of PPM1D protein may be critically important in determining the cellular response to transcription-impairing DNA damage. McKay *et al.* (50) have shown that, following exposure to UV, the mRNA levels of smaller, generally pro-apoptotic p53 target genes increased whereas the mRNA levels of larger p53 target genes, including MDM2 and PPM1D, did not change or decreased. Thus, since PPM1D phosphatase activity has been shown to reduce p38 MAPK and p53 activities in response to UV (12), the p53-dependent increase in *PPM1D* transcripts with short 5′ UTRs may allow PPM1D to function as a tissue-specific survival factor.

Control of *PPM1D* transcription by p53 and CREB/ATF family transcription factors

Transcription of the *PPM1D* gene is regulated by both p53 and CREB. We have provided evidence that CREB contributes to basal transcription of *PPM1D* and, in response to IR, that p53 is required for induced *PPM1D* expression. Although CREB and p53 each affect the transcription of hundreds of genes (18,73), the transcriptional programs regulated by the two factors are largely distinct. CREB is implicated in the response to growth and differentiation signals (17,46,74), whereas the genes regulated by p53 function in cell cycle arrest, apoptosis and DNA repair pathways (75). The deregulation of key genes that are controlled by both pathways may contribute to tumor progression. CREB and p53 have opposed effects on the

transcription of the genes for Cyclin A and BRCA1 (76–78). That CREB and p53 act in the same direction in their regulation of *PPM1D* expression may be rationalized by the negative regulation of p53 by PPM1D through dephosphorylation of p38 MAPK and ATM (11,15). Functional linkage between the p53 and CREB/ATF pathways also was suggested by the induction of p53 in cells transfected with CRE-decoy oligonucleotides (79) and by the characterization of protein–protein interactions between p53 and CREB (19).

The complex promoter of the *PPM1D* gene contains identifiable binding sites for many transcription factors that may control its transcription in particular cellular or functional contexts. The regulation of *PPM1D* expression by E2F1 recently was reported (38). The presence of a conserved CRE in the promoter of *PPM1D* together with data presented here suggest that basal transcription of the *PPM1D* gene is regulated, in part, by CREB or related family members. Although CREB activates transcription through CBP-mediated histone acetylation, the localization of functional CREs within 50 to 150 bp of the TSS (46) suggests that the direct interaction between bound CREB and transcription complexes is an important aspect of the regulation of transcription by CREB. CBP also interacts with transcription complexes and may function as a bridge between CREB and other factors (80). ATM-dependent phosphorylation of CREB at Ser111 and Ser121 following exposure to IR inhibits the interaction between CREB and CBP (81), thus reducing its capacity as a transcriptional activator. In contrast, ATM-dependent signaling to wild-type p53 in response to IR results in its stabilization and activation (82). Binding of p53 to p53RE within the *PPM1D* promoter increases transcription and shifts the position of transcription initiation. Ultimately, the increased levels of PPM1D reduce stress-induced phosphorylation of target proteins, allowing for the recovery of normal function.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

FUNDING

The Intramural Research Program of the National Institutes of Health, Center for Cancer Research, National Cancer Institute; a Laboratory Directed Research and Development Award at the Brookhaven National Laboratory and the Low Dose Program of the Office of Biological and Environmental Research of the US Department of Energy (to C.W.A.).

Conflict of interest statement. None declared.

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