A Novel p53 Transcriptional Repressor Element (p53TRE) and the Asymmetrical Contribution of Two p53 Binding Sites Modulate the Response of the Placental Transforming Growth Factor-β Promoter to p53*

Jeffrey Wong, Pei-Xiang Li, and Henry J. Klamut‡

From the Division of Experimental Therapeutics, Ontario Cancer Institute, Princess Margaret Hospital, University Health Network and the Department of Medical Biophysics, University of Toronto, Toronto, Ontario M5G 2M9, Canada

Previous studies in our laboratory and others identified placental transforming growth factor-β (PTGF-β) as an important downstream mediator of DNA damage signaling and a transcriptional target of p53. Here we show that accumulation of PTGF-β mRNA in response to p53 overexpression is delayed relative to p21WAF1, whereas the promoters of these genes respond to p53 with similar kinetics. Mutational analyses of two p53 binding sites within the PTGF-β promoter revealed that site p53-1 (−29 bp) is responsible for as much as 80% of the transcriptional response to p53. This is consistent with electrophoretic mobility shift assays showing that site p53-1 binds p53 with a much higher affinity than site p53-2 (−850 bp). We also describe for the first time a novel 242 bp sequence was shown to suppress p53 transactivation in a position- and promoter-independent manner and to associate with a 28-kDa protein expressed in several tumor cell lines. A p53 suppressor element and asymmetric p53 binding sites may participate determining the activation thresholds of p53-responsive promoters in a cell- and context-specific manner.

The p53 tumor suppressor is often referred to as the “guardian of the genome” because of its central role in regulating the cellular response to DNA damage (1). The rapid and substantial increase in p53 protein levels triggered by a genotoxic insult can be attributed primarily to stabilization of the normally labile protein (2). The ability of p53 to suppress transformation is largely dependent on its ability to act as a sequence-specific transcription factor (3). p53 in the nucleus associates with cis-acting DNA sequences composed of two copies of the palindromic motif 5′-PuPuPuCaA(T/A)GPy-PyPyPy-3′, separated by a variable linker region of between 0 and 13 bases (4). Studies in lower eukaryotes have led to the estimate that the human genome contains more than 200 genes that are directly responsive to p53 (5). Promoter mapping and microarray studies have already implicated p53 in the regulation of more than 100 genes involved in cellular processes such as DNA repair, angiogenesis, signal transduction, and oxidative stress (6). It is clear that p53 is at the helm of a diverse collection of downstream effectors.

The most intensively studied outcomes of p53 activation are cytostasis and apoptosis (7). A reversible cell cycle arrest response has been largely attributed to p53-dependent up-regulation of the cyclin-dependent kinase inhibitor p21WAF1 (8, 9). p53 has also been implicated in the induction of a premature senescence program in some cell types (10). The mechanisms underlying p53-mediated apoptosis are not as clearly defined, although a multitude of p53 target genes have been implicated in this process (11). However, no single gene appears to be crucial in p53-mediated apoptosis, and it is likely that particular subsets of downstream effectors are activated in a cell- and context-specific fashion, with each contributing in varying degrees to the overall apoptotic response (11). The propensity of p53 to initiate cell cycle arrest and apoptosis varies among different cell types (12, 13). Tumor-associated mutations in the DNA binding and hinge domains of p53 have been shown to have different effects on the ability of mutant p53 to activate p53-responsive promoters and can even lead to the specific loss of the apoptotic function of p53 but not its cell cycle arrest activity (14–17). Oda et al. (18) demonstrated that transactivation of the apoptosis-inducing gene, p53AIP, by wild-type p53 (phosphorylated at Ser-46) occurs prior to the onset of apoptosis but not cell cycle arrest. Together, these observations allude to transcriptional mechanisms that permit the differential regulation of genes downstream of p53 involved in arrest and apoptosis.

Much attention has focused on post-translational modifications within the amino- and carboxyl-terminal domains of p53 and their impact on the ability of p53 to transactivate target genes (19). Phosphorylation events within the amino-terminal activation domain have been shown to regulate p53 binding to associated proteins such as Mdm2 and components of the transcription initiation complex (20). More recently, up-regulation of p53 target genes in vivo has been shown to be contingent on acetylation of the carboxyl-terminal domain of p53 (21–23). Espinosa and Emerson (22) showed that acetylation of p53 can significantly enhance associations with transcriptional coactivators p300 and TRAP necessary for acetylation of nucleosomes in the promoters of p53 target genes. Consistent with these findings, binding and deacetylation of p53 by the NAD-dependent deacetylase SIR2α were shown to repress p53-mediated transcription and the cellular response to DNA damage.

* This work was supported by grants from the Canadian Institutes for Health Research, the Canadian Breast Cancer Foundation, and an Ontario Graduate Scholarship in Science and Technology fellowship (to J. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Medical Biophysics, University of Toronto, Ontario Cancer Institute, Princess Margaret Hospital, 610 University Ave., Rm. 10-721, Toronto, Ontario M5G 2M9, Canada. Tel.: 416-946-2981; Fax: 416-946-2984; E-mail: hklamut@uhnres.utoronto.ca.
(24, 25). Several other p53-interacting proteins have been shown to enhance (e.g. BRCA1 (26), EML (27)) or attenuate (e.g. ATF3 (28), S100B (29)) p53-mediated transcription. Among these, Samuels-Lev et al. (30) demonstrated that ASPP proteins could specifically enhance the transcription of p53 target genes involved in apoptosis, whereas Stros et al. (31) found that high mobility group B1/B2 proteins down-regulate the apoptosis-inducing Box gene in a cell-specific manner. Because none of these factors have been shown to associate directly with DNA, it is likely that they exert their effects by enhancing or interfering with the ability of p53 to bind DNA or to interact with other important trans-acting factors (e.g. Sp1 (32)) associated with p53-responsive genes. Although it is clear that promoter-specific sequence elements play an essential role in the response of individual genes to p53 activation, the nature of these regulatory elements and the mechanisms used to modulate the response of individual gene promoters to p53 are poorly understood.

Previous studies in our laboratory identified the Placental Transforming Growth Factor-β (PTGF-β)1 gene as responding to both p53-dependent and p53-independent DNA damage-signaling events (33). Overexpression of PTGF-β alone was sufficient to suppress growth and induce apoptosis of MDA-MB-468, MCF-7, and various other breast cancer cell lines, but not untransformed cell lines. We demonstrated that a 1,015-base pair region encompassing the 5'-end of the PTGF-β gene contains a functional p53 binding site (p53-1) within the 5'-untranslated region. Coincident with our study, Tan et al. (34) also identified the PTGF-β gene as p53-responsive and described a second p53 binding site (p53-2) almost 900 bp upstream of site p53-1. Here we compare the kinetics of PTGF-β promoter activity and p53 binding to sites p53-1 and p53-2, and search for cis-acting sequence elements that participate in the PTGF-β promoter response to p53.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Conditions for growth of MDA-MB-468 and MCF-7 human breast cancer cells and HeLa human cervical carcinoma cells have been described previously (35). Procedures for the growth and maintenance of adenoviral vectors have also been described previously (33).

**Northern Blotting**—Total RNA was extracted from MDA-MB-468 cells using the RNeasy kit (Qiagen, Chatsworth, CA) at 0, 12, 24, and 36 h postinfection with Adp53 (100 pfu/cell). 10 μg of each sample was analyzed by electrophoresis on 1.2% agarose-formaldehyde denaturing gels. Northern blot analysis was performed essentially as described elsewhere (36).

**Reporter Constructs and Luciferase Assays**—The p21-lux luciferase reporter construct was graciously provided by Dr. S. Benchimol (De-Elsewhere (36). The p21-lux reporter construct was analyzed by electrophoresis on 1.2% agarose-formaldehyde denaturing gel to verify that nucleotides were removed by gel filtration using Sephadex-G25 fine spin columns (Roche). Oligonucleotides were resuspended in water at ~350 μM, and left to cool to room temperature overnight. Oligonucleotides (forward sequence only is indicated): p53-1, forward primer P53B75SS (5′-GAC AGG AAC TGG GGA CCC GCC CAA-3′); reverse primer P53B75RS (5′-CTC TCC CTC TGG GCA CAA ACC TCC-3′); MTp53-1, forward primer P53MTFWS (5′-CAG TCG ACC GAG GCA AAT GTA CAC GCA-3′); p53-2, forward primer P53NTWFA (5′-TGC CAT CTT GCC CAC TCT TGG GCA-3′); MTP53-2, forward primer P53NTMTR (5′-TGG TCT GCC CCA TAC TGC CCA-3′); MTP53-3, forward primer P53NTSWS (5′-AGA TAA CTT CTC TCC TTC TTC-3′). Sequences included in oligonucleotides R5, R4, R3, R2, R1, and R are depicted in Fig. 6A. Oligonucleotide R5 corresponds to the sequence in forward primer GH724.751F (5′-AGA TTA CCA GAG GGA ATG AGG GAA-3′). 

1 The abbreviations used are: PTGF-β, placental transforming growth factor-β; CMV, cytomegalovirus; EMSA, electrophoretic mobility gel shift assay; p53TRE, p53 transcriptional repressor element; PIG-3, p53-induced gene-3; p21, plaque-forming units; ASSP, apoptosis-stimulating protein of p53; GML, glycosylphosphatidylinositol-anchored molecule-like protein.

The primers for pMT1/2 were generated using pMT2 as a template with forward primers SPFWT1 and reverse primers SPF53R. 5′-Promoter deletion constructs were generated using reverse primer SWPTR1 and the following forward primers (see Fig. 4, A and B). The pWT1Δ181 primer was STDL1500F (5′-GGTT ACC CAG CTC ATA TCG AGG AGG-3′) and pWT1Δ667 primer was STDL300F (5′-GGTT ACC CAG CTC ATA TCG AGG AGG-3′) and pWT1Δ132 primer was STDL900F (5′-GGTT ACC CAG CTC ATA TCG AGG AGG-3′). pDLPFR (see Fig. 6) was generated using a four-step cloning procedure as follows. The forward primers SPFWT1 and DLBTR (5′-GGTT AGG GCC GCC GAC AGT ATT ATG TGT AGG-3′) were used in a PCR to generate fragment C, and forward primers DLPTR (5′-GGTT GAT AGC TAA AGG AGA A-3′) and SWPTR1 were used to generate fragment D. Both fragments were cloned separately into pCR2.1-TOPO cloning vector (Invitrogen). Fragment D was cloned into the EcoRV HindIII restriction sites downstream of fragment C, and the resulting SacI/XhoI fragment containing C and D was then cloned into pGL3-Basic.

pDCLR was generated in a fashion similar to that for pDLPFR; however, primer PCRDCAF (5′-GCC GAT CTC ATA TCG AGG AGG-3′) was used instead of DLPTR, and pDLPFR was replaced by PCDCDF (5′-GCC TTC TTT TTC TGG GAG-3′). pWT-TRE2 was generated by cloning a 171-bp PCR fragment corresponding to the region between ~81 and ~251 bp using forward primer OLIT16F (5′-GGTT CTC CTC AAC AAA GAC TCC CAC-3′) and reverse primer OLIT16R (5′-GGTT ACC CTC CAG ACC GCA AAC AAC-3′) into the KpnI site in pWT. p21-TRE2 was generated in a similar fashion. All reporter constructs were sequenced using vector- and plasmid-specific primers on a LI-COR model 400 automated sequencer (ACGT Corp., Lincoln, NE). Luciferase reporter constructs were cotransfected into MDA-MB-468 cells with a constitutively expressed β-galactosidase reporter plasmid (CMV-β-galactosidase) using a calcium phosphate precipitation method (37). Luciferase was quantitated using the Dual-Light kit (PE Biosystems/Tropix) using a Berthold Lumat LB9507 luminometer and normalized to β-galactosidase activity measured in the same fashion.

**Nuclear Protein Extracts and Recombinant p53**273–290—Nuclear protein extracts from MCF-7, MDA-MB-468, or HeLa cells were prepared as described previously (38). A histidine-tagged, truncated recombinant p53 protein (amino acids 82–360, denoted p53(82–360) was expressed in Escherichia coli and purified as described previously (33).

**Electrophoretic Mobility Gel Shift Assay (EMSA) and UV-Cross-linking Assay**—Complementary, single-stranded DNA oligonucleotides were combined at 2.5 μM and end-labeled with [γ-32P]ATP. Excess nucleotides were removed by gel filtration using Sephadex-G25 fine spin columns (Roche). Oligonucleotides were resuspended in water at ~350 μM, and left to cool to room temperature overnight. Oligonucleotides (forward sequence only is indicated): p53-1, forward primer P53B75SS (5′-GAC AGG AAC TGG GGA CCC GCC CAA-3′); reverse primer P53B75RS (5′-CTC TCC CTC TGG GCA CAA ACC TCC-3′); MTp53-1, forward primer P53MTFWS (5′-CAG TCG ACC GAG GCA AAT GTA CAC GCA-3′); p53-2, forward primer P53NTWFA (5′-TGC CAT CTT GCC CAC TCT TGG GCA-3′); MTP53-2, forward primer P53NTMTR (5′-TGG TCT GCC CCA TAC TGC CCA-3′); MTP53-3, forward primer P53NTSWS (5′-AGA TAA CTT CTC TCC TTC TTC-3′). Sequences included in oligonucleotides R5, R4, R3, R2, R1, and R are depicted in Fig. 6A. Oligonucleotide R5 corresponds to the sequence in forward primer GH724.751F (5′-AGA TTA CCA GAG GGA ATG AGG GAA-3′). Conditions for
To investigate whether this induction profile could be recapitulated at the transcriptional level, the PTGF-β and p21\(^{WAF1}\) promoters were cloned into luciferase reporter vectors to generate pWT and p21-lux, respectively, and their response to p53 was examined. The PTGF-β promoter in pWT contains two functional p53 binding sites: site p53-1 at +29 bp within the 5′-untranslated region and site p53-2 at −850 bp (33); p21-lux contains 2.4 kb of the p21\(^{WAF1}\) promoter (40). MDA-MB-468 cells were transiently transfected with either the p21-lux or pWT reporter, and luciferase assays were performed at 6, 12, 18, and 24 h postinfection with Adp53. As shown in Fig. 1B, induction levels of the two promoters were virtually identical at 12 h. By 18 h the p21\(^{WAF1}\) promoter displayed near maximal 15-fold induction levels and remained at this level at the 24 h time point. Induction of the PTGF-β promoter proceeded at a slower rate, reaching 6-fold by 18 h and 13-fold by 24 h postinfection. No induction was observed in cells infected with a control adenovirus expressing the β-galactosidase gene (data not shown). Thus, although p53-mediated induction of the PTGF-β promoter is somewhat delayed relative to the p21\(^{WAF1}\) promoter at 18 h, induction levels are comparable by 24 h, consistent with the levels of endogenous mRNA transcripts observed at this time point. The absence of a significant difference in promoter induction levels at 12 h suggests that the delay in endogenous PTGF-β transcript accumulation at this time point is not caused by differences in the transcriptional response of these promoters to p53.

**Binding Sites p53-1 and p53-2 Make Distinct Contributions to the p53 Response of the PTGF-β Promoter**—To examine the contributions of p53 binding sites p53-1 and p53-2 to PTGF-β promoter responsiveness, we examined the activity of promoter constructs bearing mutations in each site. As depicted in Fig. 2A, mutant p53 binding sites were created by nucleotide substitutions (lowercase) within the core (shaded) of each 10-bp consensus repeat motif. Mutations of this nature have been shown to be sufficient to abolish p53 binding (4). Luciferase assays were performed 24 h postinfection of MDA-MB-468 cells with Adp53. As shown in Fig. 2B, mutation of site p53-1 resulted in a large decline in p53 induction: from 24-fold in pWT to 16-fold (pMT2) after Adp53 infection. Mutation of both sites (pMT1/2) completely abolished p53 responsiveness. This was confirmed in an analysis of the p53 response of constructs with a mutated p53-1 and progressively longer deletions from the 5′-end of the PTGF-β promoter (Fig. 2C). These results are consistent with the notion that site p53-1 makes a larger contribution to the p53 response than p53-2, and that the combined contribution of these sites to PTGF-β promoter induction is additive.

**Sites p53-1 and p53-2 Bind p53 with Different Affinities**—We hypothesized that the differential contribution of sites p53-1 and p53-2 might be a function of their relative binding affinities for p53. As shown in Fig. 3A, a 26-bp, double-stranded, radiolabeled oligonucleotide containing site p53-1 associates strongly with wild-type p53 in nuclear extracts derived from Adp53-infected (100 pfu/cell) MDA-MB-468 cells (lane 3) but not with endogenous mutant p53 in uninfected cells (lane 2). Binding of p53-1 by p53 was specific because competition for binding of this oligonucleotide (MTp53-1; 20× concentration) by p53 was not observed (lane 4). Furthermore, a p53 antibody (α-p53) was seen to supershift EMSA complexes generated by either p53-1 (lane 10) or a p53 consensus oligonucleotide (lane 21). Unlike site p53-1, no p53-specific EMSA band was observed with the p53-2 probe (lanes 11–14). Moreover, excess unlabeled p53-2 oligonucleotide did not compete for site p53-2, and the binding of p53 to this oligonucleotide was not supershifted by a p53 antibody (lanes 15–17). Thus, the relative binding affinity of sites p53-1 and p53-2 for p53 is consistent with their relative contributions to p53 responsiveness, with site p53-1 being more strongly bound by p53 than site p53-2.

**The PTGF-β Promoter Exhibits an Immediate-Early Response to p53 Activation**—Microarray studies in colon and lung carcinoma cell lines overexpressing wild-type p53 (6, 39) have implicated at the transcriptional level, the PTGF-β promoter is somewhat delayed relative to the p21\(^{WAF1}\) promoter at 18 h, induction levels are comparable by 24 h, consistent with the levels of endogenous mRNA transcripts observed at this time point. The absence of a significant difference in promoter induction levels at 12 h suggests that the delay in endogenous PTGF-β transcript accumulation at this time point is not caused by differences in the transcriptional response of these promoters to p53.

**DNA binding reactions and EMSAs have been described previously (38). For UV cross-linking assays, incubation reactions containing oligonucleotides and nuclear protein extracts were exposed to 8,000 J of UV radiation in a Stratalinker (Stratagene) and fractionated on a 20% Tris-glycine SDS-polyacrylamide gel for 2 h at 100 V. All gels were dried and exposed to x-ray film overnight.**

**RESULTS**

**To examine the contributions of p53 binding sites p53-1 and p53-2 to PTGF-β promoter responsiveness, we examined the activity of promoter constructs bearing mutations in each site. As depicted in Fig. 2A, mutant p53 binding sites were created by nucleotide substitutions (lowercase) within the core (shaded) of each 10-bp consensus repeat motif. Mutations of this nature have been shown to be sufficient to abolish p53 binding (4). Luciferase assays were performed 24 h postinfection of MDA-MB-468 cells with Adp53. As shown in Fig. 2B, mutation of site p53-1 resulted in a large decline in p53 induction: from 24-fold in pWT to 16-fold (pMT2) after Adp53 infection. Mutation of both sites (pMT1/2) completely abolished p53 responsiveness. This was confirmed in an analysis of the p53 response of constructs with a mutated p53-1 and progressively longer deletions from the 5′-end of the PTGF-β promoter (Fig. 2C). These results are consistent with the notion that site p53-1 makes a larger contribution to the p53 response than p53-2, and that the combined contribution of these sites to PTGF-β promoter induction is additive.**

**Sites p53-1 and p53-2 Bind p53 with Different Affinities**—We hypothesized that the differential contribution of sites p53-1 and p53-2 might be a function of their relative binding affinities for p53. As shown in Fig. 3A, a 26-bp, double-stranded, radiolabeled oligonucleotide containing site p53-1 associates strongly with wild-type p53 in nuclear extracts derived from Adp53-infected (100 pfu/cell) MDA-MB-468 cells (lane 3) but not with endogenous mutant p53 in uninfected cells (lane 2). Binding of p53-1 by p53 was specific because competition for binding of this oligonucleotide (MTp53-1; 20× concentration) by p53 was not observed (lane 4). Furthermore, a p53 antibody (α-p53) was seen to supershift EMSA complexes generated by either p53-1 (lane 10) or a p53 consensus oligonucleotide (lane 21). Unlike site p53-1, no p53-specific EMSA band was observed with the p53-2 probe (lanes 11–14). Moreover, excess unlabeled p53-2 oligonucleotide did not compete for site p53-2, and the binding of p53 to this oligonucleotide was not supershifted by a p53 antibody (lanes 15–17). Thus, the relative binding affinity of sites p53-1 and p53-2 for p53 is consistent with their relative contributions to p53 responsiveness, with site p53-1 being more strongly bound by p53 than site p53-2.

**The PTGF-β Promoter Exhibits an Immediate-Early Response to p53 Activation**—Microarray studies in colon and lung carcinoma cell lines overexpressing wild-type p53 (6, 39) have suggested that PTGF-β is induced with kinetics that are characteristic of an immediate-early p53 target gene. Northern blot analyses were employed to compare more definitively the steady-state levels of PTGF-β and p21\(^{WAF1}\) transcripts in MDA-MB-468 cells at various times after infection with a recombinant adenovirus expressing wild-type p53 (Adp53) at 100 pfu/cell. As shown in Fig. 1A, p21\(^{WAF1}\) transcripts were detected by 12 h and peaked by 24 h postinfection with Adp53. PTGF-β mRNA was not detectable at 12 h but also peaked by 24 h postinfection.
FIG. 2. Functional analysis of the contributions of p53 binding sites 1 and 2 to PTGF-β promoter induction. A, the sequences and positions of p53 binding sites p53-1 and p53-2 within the PTGF-β gene are shown below the consensus p53 binding site, which consists of a 10-base repeat separated by a spacer region 0–13 bp in length. Shaded regions represent highly conserved core nucleotides. Solid circles denote nucleotides that deviate from the consensus. Sequences of mutant PTGF-β p53 binding sites (MT5p53-1 and MT5p53-2) introduced into PTGF-β promoter constructs are shown below their respective wild-type counterparts. Lowercase letters indicate nucleotide substitutions used to generate mutations. R, purine; Y, pyrimidine; W, adenine or thymine. B, functional analysis of PTGF-β promoter constructs (+49 to −966 bp) mutated for p53 site 1 alone (pMT1), p53 site 2 alone (pMT2), or sites 1 and 2 together (pMT1/2). pWT represents the wild-type (+49 to −966 bp) PTGF-β promoter construct. Luciferase activities were determined 24 h postinfection of MDA-MB-468 cells with Adp53 (100 pfu/cell). The results are expressed as the mean fold induction ± S.E. relative to pMT1/2. C, functional analysis of PTGF-β promoter fragments mutated for site p53-1 and having successive deletions (to −673, −389, and −105 bp) from the 5'-end (−966 bp) of pWT. Luciferase activities were determined as above, and the results are expressed as the mean fold induction ± S.E. relative to pMT1/2.

for p53 binding to a consensus probe (lanes 18–20) (38). These results suggest that the affinity of site p53-1 for p53 is much higher than site p53-2 under our EMSA binding conditions.

Previous work by Tan et al. (34) suggested that p53 binding to each of these sites was dependent on the presence of monoclonal antibody Ab421 in EMSA binding reactions. As shown in Fig. 3B, inclusion of Ab421 in EMSA binding reactions produced a p53-specific band with the p53-2 probe (lane 9) which could be effectively competed by a 10- and 100-fold excess of p53-1 (lanes 10 and 11) but not by a mutant form of p53-2 (lanes 12 and 13). This EMSA complex was also supershifted by an antibody specific for the amino terminus of p53 (lane 14). These results confirmed that site p53-2 is also a specific target for wild-type p53 binding.

The intensity of bands shifted by both p53-1 and p53-2 in the presence of Ab421 (Fig. 3B, lanes 3 and 9, respectively) were comparable, suggesting that p53 is able to bind to these sites with equal affinity. To test this hypothesis, we performed EMSA experiments using a constant amount of a purified recombinant form of p53 (amino acids 82–360) lacking the carboxyl-terminal regulatory region in the presence of varying amounts of poly(dI-dC). Carboxyl-terminal truncations of p53 are known to mimic native p53 in the presence of Ab421 (41). As shown in Fig. 3C, recombinant p53 binding to the p53-1 probe could be detected in the presence of the relatively high concentrations of poly(dI-dC) used (lanes 2–5). On the other hand, recombinant p53 binding to p53-2 was only seen in the presence of low concentrations of poly(dI-dC) (lanes 7–10). This result provided further support for the notion that site p53-1 binds p53 with a much higher affinity than p53-2. This is consistent with our EMSA studies (Fig. 3A) and suggests that...
site p53-1 has a more prominent role in p53-mediated induction of PTGF-β gene expression.

Localization of a Cis-acting Sequence Element That Down-regulates the PTGF-β Promoter Response to p53—To investigate whether other regions within the PTGF-β promoter are involved in p53 induction, a series of 5’-end deletion constructs were generated and tested for responses to infection of MDA-MB-468 cells with a recombinant adenovirus expressing wild-type p53 (p53TRE). Results were reported as the mean fold induction ± S.E. as a percentage of the full-length (pWT) PTGF-β promoter. The shaded area denotes a 284-bp negative regulatory region located between −105 and −389 bp. Fine deletion analysis of the 284-bp negative regulatory region identified a 36-bp region (shaded) between −216 and −251 bp which can suppress p53-mediated activation of the PTGF-β promoter. Results are expressed as the mean fold induction ± S.E. relative to pWTΔ389.

EMSA Complex Formation Mediated by the p53TRE—To determine whether one or more trans-acting factors specifically associate with the p53TRE, a 40-bp double-stranded oligonucleotide corresponding to sequences between −212 and −251 bp (R) was end labeled and used as a probe (lane 1, probe alone) in EMSA binding reactions containing nuclear extracts prepared from MDA-MB-468 cells either untreated (lanes 2–8) or 24 h post-treatment with Adp53 (100 pfu/cell; lanes 9–15). EMSA competition experiments were performed with a 1-, 10-, or 100-fold molar excess of an unlabeled double-stranded oligonucleotide corresponding to the entire negative regulatory domain (R; lanes 3–5, 10–12) or mutant site p53-2 (MTp53-2; lanes 6–8, 13–15). Relative positions of EMSA complexes R1 (nonspecific) and R2 (specific) are indicated.

To delimit sequence elements involved in band R2 formation, a series of EMSA competition experiments were performed using oligonucleotides containing different portions of the p53TRE. The results, summarized in Fig. 6A, demonstrate that six overlapping oligonucleotides tested, only oligonucleotide Rα, containing sequences from −228 to −238 bp, did not show some level of competition for band R2 formation (Fig. 6B, lanes 11–14). Oligonucleotides Rβ and Rδ, which share a 21-bp overlap region between −222 and −242 bp, were the most effective competitors (Fig. 6B, lanes 15–18 and 23–26), followed by Rγ (lanes 19–22), Rε (lanes 27–30), and Rρ (lanes 31–34). Oligonucleotides Rα and Rβ define a 6-bp overlap region between −222 and −228 bp which appears to be necessary for R2 complex formation. Oligonucleotides Rε and Rρ demonstrate that sequences at the 5’-end (−232 to −242 bp) of the 21-bp region defined by oligonucleotide Rε also contribute to R2 complex formation. Reciprocal experiments using oligonucleotide Rε as the probe (Fig. 6B, lanes 35–44) resulted in the formation of two complexes that appear to be identical to those formed with oligonucleotide R. Complex R2 was competed efficiently by unlabeled oligonucleotide Rδ (lanes 37–40) but not by oligonucleotide RMT, in which sequences within oligonucleotide Rα are scrambled (lanes 41–44). Together, these results define a novel 21-bp p53TRE (−222 to −242 bp) that mediates the formation of a specific DNA-protein complex involved in modulating transactivation of the PTGF-β gene by p53.

The p53TRE Functions in a Position-independent Manner—To investigate the effect that disruption of the p53TRE has in the context of the full-length PTGF-β promoter, promoter constructs with mutations spanning the p53TRE (pDLPTR) or within the p53TRE (pDCR) were generated (Fig. 7). Mutant p53TRE sequences in pDCR are identical to oligonucleotide RMT, which was unable to compete efficiently for EMSA complex R2 (Fig. 6). Promoter responses were compared with that of the intact promoter (pWT) in MDA-MB-468 cells 24 h after treatment with Adp53. In each case, mutations involving the p53TRE region resulted in a significant increase in promoter induction by p53 relative to the wild-type promoter (Fig. 7).
To determine whether the p53TRE can function in a position-independent manner, a 342-bp fragment containing two copies of the 171-bp region between −81 and −251 bp was cloned upstream of the 966-bp PTGF-β promoter. As shown in Fig. 7, the addition of p53TRE elements upstream of site p53-2 (pWT-TRE2) reduced p53 responsiveness to 68% of the parental pWT construct. Similarly, the addition of p53TREs to the 5′-end of the p21WAP1 promoter (p21-TRE2) reduced p53 activation to 58% of the parental p21WAP1 promoter construct. These observations suggest that p53TRE sequences can modulate p53-dependent transcriptional activation in a position-and promoter-independent manner.

The analysis of an 8.8-kb region upstream of the PTGF-β gene transcriptional start site (GenBank accession no. AF305420) revealed the existence of candidate p53TRE sequences at −8,637, −8,194, −7,436, −5,253, and −2,320 bp. Interestingly, such sequences were situated adjacent to putative p53 binding sites identified at −8,594, −8,318, −7,562, −4,888, and −3,957, suggesting a possible functional association. Analysis of other p55-responsive genes for the presence of sequences homologous to the p53TRE identified p53TRE elements within the 14-3-3σ, PIG-3, and proliferating cell nuclear antigen genes, and single sites within the p21WAP1 and GML promoters (Table I). Alignment of these 10 candidate elements with the p53TRE at −242 bp of the PTGF-β promoter identified a putative consensus binding region containing a highly conserved 11-bp core sequence (CCCAGCCTGGA). No matches to the 21-bp p53TRE were obtained in a search of the vertebrate matrix group of the TRANSFAC 5.0 database (transfac.gbf.de/TRANSFAC/) using MatInspector Professional software (genomatix.gsdf.de).

The p53TRE Associates with a 28-kDa Trans-acting Factor Expressed in Several Human Tumor Cell Lines—To examine whether trans-acting factors specific for the p53TRE are expressed in other cell types, nuclear extracts prepared from MCF-7 and HeLa cells were used in EMSA binding reactions containing the oligonucleotide R probe. As shown in Fig. 8A, complex R2 formation was observed in all EMSA binding reactions (lanes 2–7), suggesting that transcription factors specific for the p53TRE are widely expressed. UV cross-linking the transcription factors specific for the p53TRE were identified by fractionation of UV-cross-linked binding reactions on SDS-polyacrylamide gels, identified three distinct protein-DNA complexes estimated to be 180, 90, and 28 kDa in size (Fig. 8B, lanes 2–4). Because bands at 180 and 90 kDa were also observed in parallel experiments containing oligonucleotide p53-1 (lane 6), these were considered to be nonspecific. Only the 28 kDa band was specific for binding reactions mediated by the p53TRE oligonucleotide, implicating this p53TRE-binding protein (p53TRE-BP) in the attenuation of PTGF-β promoter transactivation by p53.
p53 is a sequence-specific transcription factor that regulates the expression of multiple genes; many are involved in the induction of cell cycle arrest and apoptosis in response to DNA damage. p53 functions in a context- and cell-specific fashion (42). Such functional flexibility alludes to the existence of intricate mechanisms that regulate the p53-mediated transcriptional response of individual target genes. Previous studies in our laboratory (33) and others (34) have implicated PTGF-β as an important downstream target of p53-dependent DNA damage signaling. Here we demonstrate that the PTGF-β promoter responds to p53 with kinetics comparable with the p21WAF1 promoter and that this response is mediated by two p53 binding sites: a high affinity site (p53-1) at the promoter and that this response is mediated by two p55 bind-

### TABLE I
Sequences homologous to the p53TRE in p53 target genes

| Gene | Location | Sequence |
|------|----------|----------|
| PTGF-β | -8637 | GTT GCC CAG GCT GAT CTC GAA |
| | -8194 | GTC GCC CAG GCT GGA TGC GAG |
| | -7436 | GTC GCC CAG GCT GGA TGC GGT |
| | -5253 | ACT ACC CAG GCT GGA TGC GAG |
| | +2320 | GTC ACC CAG GCT GGA TGC GAG |
| p21WAF1 | -3254 | GTC ACC CAG GCT GGA TGC GAG |
| | -5393 | GTC ACC CAG GCT GGA TGC GAG |
| | -2582 | GTC ACC CAG GCT GGA TGC GAG |
| PIG-3 | -5692 | GTC ACC CAG GCT GGA TGC GAG |
| | -1360 | TGC TCC CAG GCT GGA TGC GAG |
| GML | -8749 | GTC GCC CAG GCT GGA TGC GAG |
| PCNA | +1448 | GTC ACC CAG GCT GGA TGC GAG |
| | +3644 | GTC ACC CAG GCT GGA TGC GAG |

**Consensus**

| R = Purine | S = Guanine or Cytosine |
|-----------|------------------------|
| Y = Pyrimidine | D = Guanine or Adenine or Thymine |

DISCUSSION

PTGF-β promoter induction by p53 proceeds more slowly than the p21WAF1 promoter between 12 and 18 h postinfection with Adp53, but no significant differences were observed at 12 h, and both promoters reached similar levels of induction (13-15-fold) by 24 h. This is in contrast to our Northern blot results showing that PTGF-β transcript accumulation is delayed by as much as 12 h relative to p21WAF1. Recently, Baek et al. (43) reported that the dietary phenolic compound, resveratrol, and the DNA-damaging agent, etoposide, could induce the accumulation of PTGF-β mRNA and protein in HCT-116 cells in a p53-dependent manner. Consistent with our observations, p53 was seen to accumulate as early as 3 h post-treatment, whereas increased levels of PTGF-β mRNA and protein remained undetectable until at least 20 h later. Based on our comparison of promoter activities, the apparent delay in PTGF-β mRNA accumulation relative to p21WAF1 at 12 h does not appear to involve differences in the transcriptional response of these genes to p53. Rather, this delay is likely a reflection of significantly lower levels of basal PTGF-β gene expression in these cells, with the result that PTGF-β gene transcripts require more time to accumulate to detectable levels after p53 induction. PTGF-β levels may cross the threshold of detection only in the face of sustained high level expression of p53. In support of this view we have observed that the PTGF-β promoter is much less active than the p21WAF1 promoter in the absence of p53. It is also unlikely that p53-mediated changes in factor binding at the p53TRE contribute to this delay because p53 overexpression was seen to have no effect on p53TRE binding at the 24 h time point corresponding to the peak of PTGF-β promoter induction. Rather, levels of factor binding to the p53TRE may contribute to the maintenance of low basal levels of PTGF-β gene expression and may have an important influence on the rate and maximal level of transcriptional induction of this gene by p53.

Of the two known p53 binding sites within the PTGF-β gene, site p53-1 has a much higher affinity for p53 but displays lower homology to the p53 consensus (17/20-bp match) than site p53-2 (18/20-bp match). In fact, analysis of the sequence of p53 binding sites from other p53 target genes such as p21WAF1 (40), 14-3-3r (44), and cyclin G1 (45) indicated that the degree of adherence to the consensus sequence did not accurately predict the contribution of each binding site to p53 responsiveness. Clearly, individual nucleotides, or the arrangement of specific subsets of nucleotides, must have an important influence on the affinity of these binding sites for p53. In support of this idea, Kim et al. (46) found that p53 binding sites having internal symmetry shift more readily toward a cruciform or stem-loop structure that acts as a more favorable binding substrate for p53. The existence of six potential intranadiral bp...
p53-mediated Transactivation of the PTGF-β Promoter

The existence of homologous DNA elements in p53 target genes such as p21WAF1, 14-3-3α, PIG-3, GML, and proliferating cell nuclear antigen, along with expression of a p53TRE-binding protein in at least three different cancer cell lines, is consistent with a general role for this regulatory element in modulating the cellular response to p53. Recently, a negative regulatory region within the murine and human Bax promoters was able to mute the activity of an endogenous p53 binding site (54); however, sequence analysis of this region has not revealed any homology to the p53TRE. The mechanism by which factor binding to the p53TRE inhibits the PTGF-β promoter response to p53 remains to be determined. However, it is likely that the p53TRE interferes directly with p53 binding, perhaps by tethering p53 away from its binding sites or by destabilizing stem-loop structures in p53 binding sites. Alternatively, the p53TRE-binding protein could block associations between p53 and other essential trans-acting factors (e.g. Sp1) within the PTGF-β promoter (21, 22). Because many of these cofactors associate with either the amino- or carboxyl-terminal domains of p53, it would be interesting to determine whether p53TRE function is dependent on post-translational modifications of one or both of these domains. In combination with p53 binding sites having different affinities for p53, the p53TRE could function to restrict promoter activation until a given threshold concentration of p53 is achieved within the nucleus. Suppressor elements such as the p53TRE represent a novel mechanism for the regulation of p53 transactivation thresholds at the level of individual gene promoters. Further characterization of the p53TRE-binding protein and its association with p53 and associated trans-acting factors will provide valuable insights into transcriptional mechanisms governing the cellular response to p53.

Acknowledgments—We thank C. Arrowsmith and A. Ayed for providing recombinant p53 protein, S. Benchimol for donating the p21-lux construct, and V. Skalski for help with the UV-cross-linking assays.

REFERENCES

1. May, P., and May, E. (1999) Oncogene 18, 7621–7636
2. Ashcroft, M., and Vousden, K. H. (1999) Oncogene 18, 7637–7643
3. Raycroft, L., Wu, H. Y., and Lozano, G. (1999) Science 249, 1049–1051
4. el-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W., and Vogelstein, B. (1992) Nat. Genet. 1, 45–49
5. Tokino, T., Thiagalingam, S., el-Deiry, W. S., Waldman, T., Kinzler, K. W., and Vogelstein, B. (1994) Hum. Mol. Genet. 3, 1537–1542
6. Zhao, R., Gish, K., Murphy, M., Yin, Y., Netterman, D., Hoffman, W. H., Tom, E., Mack, D. H., and Levine, A. J. (2000) Genes Dev. 14, 981–993
7. Bates, S., and Vousden, K. H. (1996) Curr. Opin. Genet. Dev. 6, 12–18
8. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J., and Lodish, P. (1995) Cell 82, 675–684
9. el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, E. W., Kinzler, K. W., and Vogelstein, B. (1993) Cell 73, 817–825
10. Lin, A. W., Barradas, M., Stone, J. C., van Aelst, L., Serrano, M., and Lowe, S. W. (1998) Genes Dev. 12, 3008–3019
11. Vogelstein, B., Lane, D., and Levine, A. J. (2000) Nature 408, 307–310
References:

12. Di Leonardo, A., Linke, S. P., Clarkin, K., and Wahl, G. M. (1994) *Genes Dev.* 8, 2540–2551
13. Radford, I. R., Murphy, T. K., Radley, J. M., and Ellis, S. L. (1994) *Int. J. Radiat. Biol.* 65, 217–227
14. Rowan, S., Ludwig, R. L., Haupts, Y., Bates, S., Lu, X., Oren, M., and Vousden, K. H. (1996) *EMBO J.* 15, 827–838
15. Ryan, K. M., Phillips, A. C., and Vousden, K. H. (2001) *Curr. Opin. Cell Biol.* 13, 332–337
16. Kong, X. T., Gao, H., and Stanbridge, E. J. (2001) *J. Biol. Chem.* 276, 32980–33000
17. Ludwig, R. L., Bates, S., and Vousden, K. H. (1996) *Mol. Cell. Biol.* 16, 4952–4960
18. Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y., and Taya, Y. (2000) *Cell* 102, 849–862
19. Lakin, N. D., and Jackson, S. P. (1999) *Oncogene* 18, 7644–7655
20. Prives, C. (1996) *Cell* 95, 5–8
21. Barlev, N. A., Liu, L., Chehab, N. H., Mansfield, K., Harris, K. G., Halazonetis, T. D., and Berger, S. L. (2001) *Mol. Cell* 8, 1243–1254
22. Espinosa, J. M., and Emerson, B. M. (2001) *Mol. Cell* 8, 57–69
23. Wang, T., Kobayashi, T., Takimoto, R., Denes, A. E., Snyder, E. L., el-Deiry, W. S., and Brachmann, R. K. (2001) *EMBO J.* 20, 6404–6413
24. Luo, J., Nikolai, A. Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., and Gu, W. (2001) *Cell* 107, 137–148
25. Vaziri, H., Dessain, S. K., Eaton, E. N., Imai, S. I., Frye, R. A., Pandita, T. K., Guarente, L., and Weinberg, R. A. (2001) *Cell* 107, 140–159
26. Zhang, H., Somasundaram, K., Peng, Y., Tian, H., Bi, D., Weber, B. L., and el-Deiry, W. S. (1998) *Oncogene* 16, 1713–1721
27. Garkavtsev, I. V., Kley, N., Grigorian, I. A., and Gudkov, A. V. (2001) *Oncogene* 20, 827–836
28. Yan, C., Wang, H., and Boyd, D. D. (2002) *J. Biol. Chem.* 277, 10804–10812
29. Lin, J., Blake, M., Tang, C., Zimmer, D. B., Rastundi, R. R., Weber, D. J., and Carrier, F. (2001) *J. Biol. Chem.* 276, 35037–35041
30. Samuel-Lee, V., O’Connor, D. J., Bergamaschi, D., Trigianti, G., Hsieh, J. K., Zhong, S., Campargue, I., Naumovski, L., Crook, T., and Lu, X. (2001) *Mol. Cell* 8, 781–794
31. Siciliano, M. C., Tchale, T., Baccakova, A., Kageyama, H., and Nakagawara, A. (2002) *J. Biol. Chem.* 277, 7157–7164
32. Koutsodontis, G., Tentes, I., Papakosta, P., Moustakas, A., and Kardassis, D. (2002) *J. Biol. Chem.* 276, 29116–29125
33. Li, P. X., Wong, J., Ayed, A., Ngo, D., Brade, A. M., Arrowsmith, C., Austin, R. C., and Klamut, H. J. (2000) *J. Biol. Chem.* 275, 20127–20135
34. Tan, M., Wang, Y., Guan, K., and Sun, Y. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 109–114
35. Li, P., Bui, T., Gray, D., and Klamut, H. J. (1998) *Breast Cancer Res. Treat.* 48, 273–286
36. Ounin, P. A., Sood, S. K., Liaw, P. C., Sarge, K. D., Maeda, N., Hirsh, J., Ribau, J., Podor, T. J., Weitz, J. I., and Austin, R. C. (1998) *Biochem. J.* 332, 213–221
37. Chen, C. A., and Okayama, H. (1988) *BioTechniques* 6, 632–638
38. Woloszczuk, R., Peled, A., Elkind, N. B., and Rotter, V. (1998) *Cancer Detect. Prev.* 22, 1–13
39. Kannan, K., Amariglio, N., Rechavi, G., Jakob-Hirsch, J., Kela I Kaminski N. Getz, G., Domany E, and Givol D. (2001) *Oncogene* 20, 2225–2234
40. el-Deiry, W. S., Tokino, T., Waldman, T., Oliner, J. D., Velculescu, V. E., Burrell, M., Hill, D. E., Healy, E., Rees, J. L., and Hamilton, S. R. (1995) *Cancer Res.* 55, 2910–2919
41. Hupp, T. R., Mee, D. W., Midgley, C. A., and Lane, D. P. (1995) *Nucleic Acids Res.* 21, 3167–3174
42. Yu, J., Zhang, L., Hwang, P. M., Rago, C., Kinzler, K. W., and Vogelstein, B. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 14517–14522
43. Baek, S. J., Wilson, L. C., and Eling, T. E. (2002) *Carcinogenesis* 23, 425–432
44. Hermetek, H., Lengauer, C., Polyak, K., He, T. C., Zhang, L., Thiagalingam, S., Kinzler, K. W., and Vogelstein, B. (1997) *Mol. Cell* 1, 3–11
45. Yardley, G., Zauberman, A., Oren, M., and Jackson, P. (1998) *FEBS Lett.* 430, 171–175
46. Kim, E., Albrechtsen, N., and Degpert, W. (1997) *Oncogene* 15, 857–869
47. Hupp, T. R., and Lane, D. P. (1994) *Curr. Biol.* 4, 865–875
48. Halazonetis, T. D., Davis, L. J., and Kandil, A. N. (1993) *EMBO J.* 12, 1021–1026
49. Hupp, T. R., Mee, D. W., Midgley, C. A., and Lane, D. P. (1992) *Cell* 71, 875–886
50. Gu, W., and Roeder, R. G. (1997) *Cell* 90, 585–606
51. Ayed, A., Mulder, F. A., Yi, G. S., Guan, K., and Arrowsmith, C. H. (2001) *Nat. Struct. Biol.* 8, 756–760
52. Kaeberlein, M. D., and Ito, R. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 95–100
53. Wouters, D., Gu, J., and Yuan, Z. M. (2001) *J. Biol. Chem.* 276, 27999–28005
54. Thornborrow, E. C., Patel, S., Mastroiopietro, A. E., Schwartzfarb, E. M., and Manfredi, J. J. (2002) *Oncogene* 21, 990–999
A Novel p53 Transcriptional Repressor Element (p53TRE) and the Asymmetrical Contribution of Two p53 Binding Sites Modulate the Response of the Placental Transforming Growth Factor-β Promoter to p53

Jeffrey Wong, Pei-Xiang Li and Henry J. Klamut

J. Biol. Chem. 2002, 277:26699-26707.
doi: 10.1074/jbc.M203020200 originally published online May 14, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M203020200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 54 references, 18 of which can be accessed free at http://www.jbc.org/content/277/29/26699.full.html#ref-list-1