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p53 Upregulates Death Receptor 4 Expression through an Intronic p53 Binding Site

Xiangguo Liu, Ping Yue, Fadlo R. Khuri, and Shi-Yong Sun

Winship Cancer Institute, Emory University School of Medicine, Atlanta, Georgia

Abstract

Death receptor 4 (DR4) is one of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors and triggers apoptosis on ligation with TRAIL or overexpression. Our previous study demonstrated that DR4 expression could be regulated in a p53-dependent fashion. In the present study, we have demonstrated that DR4 is a p53 target gene and is regulated by p53 through a functional intronic p53 binding site (p53BS) based on the following lines of evidence: (*a*) the p53BS in the *DR4* gene is almost identical to the one found in the first intron of the *DR5* gene in terms of their locations and sequences; (*b*) DR4 p53BS bound to p53 protein in intact cells upon p53 activation as demonstrated by a chromatin immunoprecipitation assay; (*c*) a luciferase reporter vector carrying the DR4 p53BS upstream of an SV40 promoter exhibited enhanced luciferase activity when transiently cotransfected with a wild-type p53 expression vector in p53-null cell lines or stimulated with DNA-damaging agents in a cell line having wild-type p53; and (*d*) when the DR4 p53BS, together with its own corresponding promoter region in the same orientation as it sits in its natural genomic locus, was cloned into a basic luciferase vector without a promoter element, its transcriptional activity was strikingly increased by cotransfection of a wild-type p53 expression vector or treatment with DNA-damaging agents. However, wild-type p53 or DNA-damaging agents completely lost their activity to increase transcriptional activity of a reporter construct with deleted DR4 p53BS. Thus, we conclude that p53 directly regulates the expression of the *DR4* gene via the novel intronic p53BS.

Introduction

For the past few decades, great efforts have been made to reveal the mechanism by which p53 regulates apoptosis. It generally is agreed that p53 primarily acts as a transcription factor and induces apoptosis by transcriptionally modulating the expression of its proapoptotic target genes, although it also induces apoptosis in a transcription-independent fashion (1, 2). It also is clear that most functional genes regulated by p53 contain classical p53 binding sites (p53BSs) in their promoter or intronic regions (3). However, unlike the cell cycle inhibitory capacity of p53, which appears to be primarily mediated by a handful of genes dominated by $p21^{WAF1}$, the apoptosis-inducing ability of p53 may involve transactivation of multiple proapoptotic genes, among which some encode death domain-containing proteins, including the death receptors Fas and death receptor 5 (DR5), whereas others encode mitochondria-related proteins (*e.g.,* Bax, Noxa, Puma, and p53AIP1), along with others having either proven or putative roles in different steps of the apoptotic cascades, such as PIGs, caspase-6, Bid, and Apaf-1 (1–3). These genes may somehow contribute to p53-mediated cell death under certain conditions, in certain specific tissues, or in different cell types. However, no single target gene is an absolute mediator for p53-dependent apoptotic cell death (3).

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; also called APO-2L), a newly identified member of the tumor necrosis factor family, recently has attracted much attention because it induces apoptosis in a wide variety of transformed cells but does not seem to be cytotoxic to normal cells *in vitro* and *in vivo* (4, 5). Therefore, TRAIL is considered to be a tumor-selective, apoptosis-inducing cytokine and a promising new candidate for cancer management (6, 7). TRAIL induces apoptosis by interacting with two death domain-containing receptors: death receptor 4 (DR4; also called TRAIL receptor 1) and DR5 (also called TRAIL receptor 2 or Killer/DR5; Refs. 6, 7). In addition, TRAIL can bind to two decoy receptors: DcR1 (also called TRAIL receptor 3) and DcR2 (also called TRAIL receptor 4). These receptors, which contain either no cytoplasmic death domain or a truncated death domain, can compete with DR4 and DR5 for TRAIL binding and thereby act as negative regulators of the effects of TRAIL (6, 7).

DR5 has been demonstrated to be induced by DNA-damaging agents in a p53-dependent fashion (8), and its transcription is directly transactivated by p53 through an intronic sequence-specific p53BS (9). Our previous work has demonstrated that DR4 also is a DNA damage-inducible, p53-regulated gene, although we did not identify a p53BS at that time in its 5′-flanking region (10). In agreement with our findings, other investigators also observed p53-dependent induction of DR4 in different systems (11–15). Interestingly, p53 also induces the expression of DcR1 and DcR2 (16, 17). It recently has been demonstrated that p53 regulates DcR1 expression through an intronic p53BS (18).

It is known that the genes encoding DR4, DR5, DcR1, and DcR2 are highly homologous and map together to a tight cluster on human chromosome 8p21–22, suggesting that they originated from a common ancestral gene (19, 20) and that their expression may be regulated through similar mechanisms (*e.g.,* p53). Therefore, we speculated that DR4 also might be regulated by p53 through a similar intronic p53BS. The availability of the DR4 genomic sequence allows us to search for just such a putative p53BS. In this study, we compared the sequences of first introns of *DR4* and *DR5* genes and identified a putative p53BS in the first intron of *DR4* gene, which is similar to the one in the first intron of *DR5* gene. Moreover, we have proved that it is a functional p53BS that mediates p53-dependent regulation of DR4 expression.

Materials and Methods

Reagents. RPMI 1640 medium with L-glutamine and fetal bovine serum was obtained from Mediatech Inc. (Herndon, VA). All of the restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). PCR reagents were purchased from Invitrogen (Carlsbad, CA). Etoposide (VP16), doxorubicin (DXR), and other chemical reagents were purchased from Sigma Chemicals (St. Louis, MO).

Cell Lines and Cell Culture. The human tumor cell lines H1299 (p53-null), H358 (p53-null), and MCF-7 (wild-type p53) were purchased from American Type Culture Collection (Manassas, VA). They were grown in monolayer culture in RPMI 1640 medium with glutamine supplemented with

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Requests for reprints: Shi-Yong Sun, Winship Cancer Institute, Emory University School of Medicine, 1365-C Clifton Road, Clinical Building C3088, Atlanta, GA 30322. Phone: 404-778-2170; Fax: 404-778-5520; E-mail: shi-yong_sun@emoryhealthcare.org.

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5% fetal bovine serum at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air.

Western Blot Analysis. Preparation of whole cell protein lysates and the procedures for the Western blot analysis were described previously (21). Whole cell protein lysates (50 μg) were electrophoresed through 10% or 12% denaturing polyacryamide slab gels and transferred to a PROTRAN pure nitrocellulose transfer membrane (Schleicher & Schuell BioScience, Keene, NH) by electroblotting. The blots were probed or reprobed with the primary antibodies, and antibody binding then was detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL) according to the manufacturer's protocol. Mouse monoclonal anti-p53 (Ab-6) antibody was purchased from EMD Bioscience (La Jolla, CA). Mouse monoclonal anti-DR4 antibody was purchased from Imgenex (San Diego, CA). Rabbit polyclonal anti-DR5 antibody was purchased from ProSci (Poway, CA). Rabbit polyclonal antihuman glyceraldehyde-3-phosphate dehydrogenase antibody was purchased from Trevigen (Gaithersburg, MD).

Adenoviral Infection. H1299 or H358 cells at a density of 2×10^6 were seeded in 10-cm cell culture dishes the day before infection. On the second day, the medium was replaced with 2.5 ml fresh serum-free RPMI 1640 medium containing 50 multiplicity of infection of adenovirus carrying wild-type 53 (Ad5-CMV-hp53) or empty vector (Ad-CMV). Cells were incubated in CO_2 incubator and shaken once every 15 min. After 2 h, 7.5 ml RPMI 1640 medium with 5% fetal bovine serum were added and incubated for 24 h. Ad5-CMV-hp53 and Ad-CMV were purchased from Qbiogene Inc. (Carlsbad, CA).

ChIP Assay. Chromatin immunoprecipitation (ChIP) assay was conducted using the ChIP assay kit purchased from Upstate Biotechnology (Lake Placid, NY) following the manufacturer's instruction. In brief, H1299 or H358 cells were infected with Ad5-CMV-hp53. After 24 h, genomic DNA and protein were cross-linked by addition of formaldehyde (1% final concentration) directly into the culture medium and incubated for 10 min at 37°C. Cells then were collected and lysed in 200 μl of SDS lysis buffer with a protease inhibitor mixture and sonicated to generate DNA fragments ~200–1000-bp long. After centrifugation, the cleared supernatant was diluted 10-fold with ChIP dilution buffer and incubated at 4°C overnight with anti-p53 monoclonal antibody (Ab-1; EMD Bioscience) or mouse $IgG_{2\alpha}$ isotype antibody (EMD Bioscience). Immune complexes were precipitated, washed, and eluted as recommended. After DNA-protein cross-linkages were reversed by heating at 65°C for 4 h, DNA was extracted in phenol/chloroform, precipitated with ethanol, and resuspended in 50 μl of 10 mM Tris-HCl and 1 mM EDTA (pH 8.0). Immunoprecipitates containing $IgG_{2\alpha}$ isotype antibody served as controls. Each sample at the same volume was used as a template for PCR amplification of fragments containing the potential p53BS on immunoprecipitated chromatin, using specific primers as follows for DR4 and DR5, respectively: DR4 sense, 5′-CTCGAGAAGTTTGTCGTCGTCGGGGT-3′; DR4 antisense, 5′-GAGCTCCCGTTCTTCCTCCGACTC-3′; DR5 sense, 5′-CTCGAGGTCCTGCTGTTGGTGAGT-3′; and DR5 antisense, 5′-GAGCTCGGGAATTTACACCAAGTGGAG-3′.

Plasmid Constructions. The 188-bp and 244-bp DNA fragments containing intronic p53BSs of *DR4* and *DR5*, respectively, were amplified from H1299 genomic DNA with PCR amplification using the primers described in the aforementioned ChIP assay. These fragments then were subcloned, respectively, into pGL3-promoter luciferase vector (pGL3-P-luc; Promega, Madison, WI), which contains an SV40 promoter upstream of the luciferase gene, through *Xho*I and *Sac*I sites. The corresponding constructs were named pP-DR4/p53BS-luc and pP-DR5/p53BS-luc, respectively. In addition, the 1030-bp DNA fragment comprising DR4 promoter region, exon 1, and partial p53BS-containing intron region and the 976-bp fragment containing DR4 promoter region, exon 1, and partial intron region without p53BS were amplified, respectively, by PCR with the following primers: DR4-BS-*Kpn*I sense, 5′-GGTACCGTAATCCCAGTTACTCAGGAGGCTG-3′; DR4-BS(+)-*Bgl*II antisense, 5′-AGATCTGACCCCGTTCTTCCTCCGACT-3′; and DR4-BS(−)-*Bgl*II antisense, 5′-AGATCTCGCCACAAGTGACCCGGGCCAGGCAC-3′. These fragments then were cloned into the pGL3-basic luciferase vector (pGL3-B-luc), which has no promoter (Promega), using *Kpn*I and *Bgl*II restriction sites. These constructs were named pB-DR4/p53BS-luc and pB-DR4/p53BS(−)-luc, respectively.

Transient Transfection and Reporter Activity Assay. The p53-luc reporter plasmid, which contains the luciferase reporter gene driven by a basic promoter element (TATA box) joined to 14 repeats of p53BSs, was purchased from Stratagene (La Jolla, CA) and used as a positive control for p53 activity. A pCH110 plasmid encoding β-galactosidase (β-gal) was purchased from Amersham (Piscataway, NJ). pCMV-p53 and pCMV-p53mt135 expression vectors, which carry wild-type *p53* and mutant *p53*, respectively, were purchased from Clontech (Palo Alto, CA). The corresponding empty vector named pCMV was made from pCMV-p53 by removing p53 sequence and religating the vector. The *p53mt135* gene differs from the *p53* gene by a G to A conversion at nucleotide 1017. pCMV-p53mt135 expresses the p53mt135 mutant, which because of a conformation change, can no longer interact with p53BSs. All of the plasmids used for transfection and luciferase assay were purified with Qiagen/Filter Plasmid Maxi Kit (Qiagen, Valencia, CA). For examining the effect of p53 overexpression on the reporter assay, cells were seeded in 24-well plates and cotransfected with the given reporter plasmid (0.133 μg/well), expression vector carrying wild-type p53, mutant p53, or no p53 (0.133 μg/well), and pCH110 plasmid (0.067 μg/well) using FuGene 6 transfection reagent (1 μl/well; Roche Molecular Biochemicals, Indianapolis, IN) following the manufacturer's protocol. For evaluating the activation of endogenous p53 by DNA-damaging agents, cells were cotransfected with the given reporter vector (0.267 μg/well) and pCH110 plasmid (0.067 μg/well) using the FuGene 6 transfection reagent (1 μl/well) and then treated with DNA-damaging agents. After incubation for the given times, cells were subjected to a luciferase activity assay using Luciferase Assay System (Promega) in a luminometer. Relative luciferase activity was normalized to β-gal activity, which was measured as described previously (22).

Results

Overexpression of p53 Up-Regulates the Expression of DR4 in p53-Null Cancer Cells. Up-regulation of the expression of DR4 by p53 has been demonstrated in different cells or systems by us (10) and others (11–15). In the current study, we also compared the effects of p53 overexpression via adenoviral infection on the expression of DR4 and DR5 in p53-null lung cancer cell lines. As shown in Fig. 1, Ad5-CMV-hp53 infection resulted in increased expression of DR4 and DR5 in H358 and H1299 cell lines evaluated by Western blot analysis. Thus, this result confirms our previous finding that DR4 is a p53-regulated gene (10).

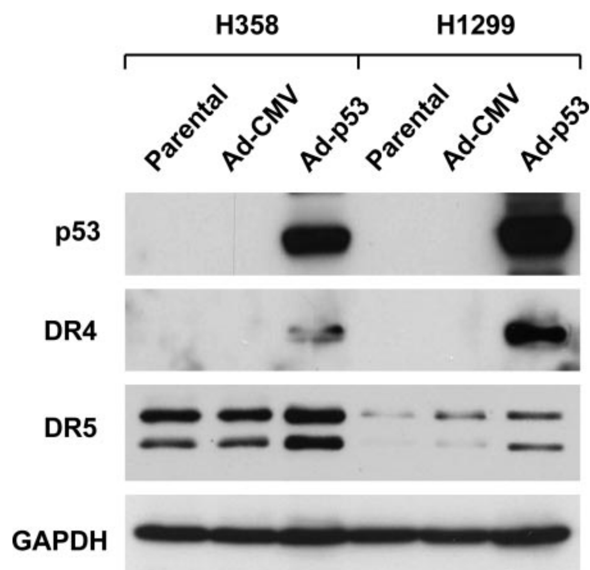

Fig. 1. Up-regulation of death receptor 4 (*DR4*) and death receptor 5 (*DR5*) expression by p53 overexpression in p53-null cancer cells. H1299 or H358 cells were infected on the second day after seeding with adenovirus carrying an empty vector (*Ad-CMV*) or wild-type *p53* gene (*Ad-p53*). After 24 h, cells were harvested for preparation of whole cell protein lysates. Western blot analysis was performed to detect the expression of p53, DR4, DR5, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) using antibodies against p53, DR4, DR5, and GAPDH, respectively.

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Sequence-Based Identification of a Putative p53BS in the First Intron of *DR4* Gene. By comparing the first intron sequences of the *DR4* and *DR5* genes, we identified a putative p53BS in the first intron of the *DR4* (DR4-p53BS) gene, which is almost identical to the one (DR5-p53BS) reported within intron 1 of the *DR5* gene (Ref. 9; Fig. 2*A*). DR4-p53BS has 80% homology with the p53 consensus DNA-binding sequence (23) and shares 90% homology with DR5-p53BS (Fig. 2*A*). Moreover, it locates only 108 bp away from the boundary of exon 1 and intron 1, which is similar to the location of DR5-p53BS, which is 109 bp away from the boundary of exon 1 and intron 1 (Fig. 2*A*), indicating that it is close to its promoter regulatory region. Considering identical locations and similar sequences between DR4-p53BS and DR5-p53BS, we speculated that this p53BS in the intron 1 of *DR4* gene was likely to be functional and to mediate transcriptional regulation of *DR4* gene expression by p53.

Detection of p53 Binding to DR4-p53BS in Intact Cells Using ChIP Assay. To determine whether p53 actually binds to DR4-p53BS in cells on p53 activation, we performed the ChIP assay to detect formation of DR4-p53BS binding complex with p53 in H358 or H1299 p53-null cell lines infected with Ad5-CMV-hp53. As a control, we also detected DR5-p53BS bound to p53. As shown in Fig. 2*B*, we detected DNA fragments containing DR4-p53BS and DR5-p53BS, respectively, from genomic DNA precipitated with p53-specific antibody but not from that pulled down with a control $IgG_{2\alpha}$ isotype antibody in either cell line. This result clearly indicates that p53 protein binds to DR4-p53BS as it does to DR5-p53BS in intact cells upon p53 activation, indicating that DR4-p53BS is a functional response element.

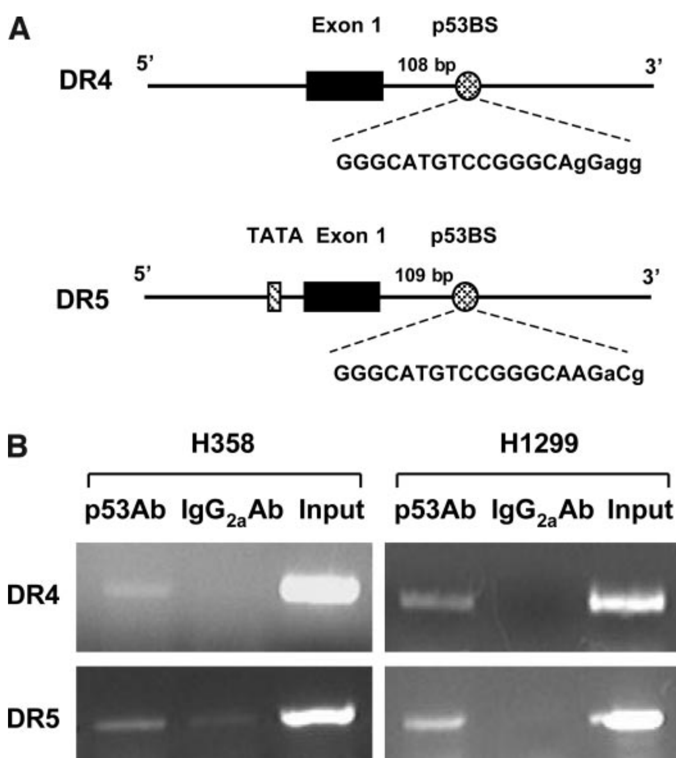

Fig. 2. Comparison of the locations and sequences of death receptor 4 (*DR4*)-p53 binding site (*p53BS*) and death receptor 5 (*DR5*)-p53BS (*A*) and detection of DR4-p53BS bound to p53 in intact cells upon p53 activation (*B*). *A*, DR4-p53BS is 80% identical to the classical p53BS that is defined as two copies of the 10-bp motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ separated by 0–13 bp (23), where Pu represents purine and Py represents pyrimidine. DR4-p53BS and DR5-p53BS also share 90% sequence homology. *B*, H358 and H1299 p53-null cell lines were infected with adenovirus carrying wild-type 53 for 24 h and then subjected to chromatin immunoprecipitation assay as described in "Materials and Methods." DR5-p53BS here was detected as a known sequence bound to p53 when p53 is activated. The amplified DNA fragments for DR4 and DR5 by PCR were 188 bp and 244 bp, respectively. *p53Ab,* p53 antibody; *IgG2aAb,* isotype antibody.

The Reporter Construct Carrying Intronic DR4-p53BS Exhibits Increased Transcriptional Activity in Response to p53 Overexpression or Treatment with DNA-Damaging Agents. To examine whether DR4-p53BS is functional to mediate p53-dependent upregulation of *DR4* gene expression, we amplified 188-bp and 244-bp intronic fragments carrying DR4-p53BS and DR5-p53BS, respectively, and cloned each of the fragments into a pGL3-promoter luciferase vector upstream of a minimal SV40 promoter (Fig. 3*A*). In this study, we used DR5-p53BS, which is known to be a functional p53BS, as a positive control for comparison (9). When the individual reporter constructs were transiently cotransfected with empty pCMV, pCMV-p53, or pCMV-p53mt135 vector into H1299 p53-null cells, we found that transfection of pCMV-p53, but not pCMV or pCMV-p53mt135, significantly increased the luciferase activity of reporter plasmid carrying either DR4-p53BS or DR5-p53BS (Fig. 3*B*). To ensure the expression of *p53* or *p53mt135* gene after transient transfection, we conducted the Western blot analysis to detect their expression in H1299 cells. As shown in Fig. 3*C*, p53 and p53mt135 expression was detected from cells transfected with pCMV-p53 and pCMV-p53mt135, respectively, indicating that p53 or p53mt135 is expressed in the tested cell line. We noted that p53mt135 level was much higher than p53 level. This could be explained by the fact that mutant p53 protein is more stable than wild-type p53 protein. Thus, these results demonstrate that overexpression of p53 enhances transcriptional activity of the reporter plasmid carrying either DR4-p53BS or DR5-p53BS. Similar results also were observed when we cotransfected these plasmids into H358 p53-null cells (data not shown). Moreover, we examined the effects of activation of endogenous p53 on transactivation of these reporter vectors by treating wild-type p53-containing cancer cells with DNA-damaging agents. In MCF-7 cells, VP16 and DXR increased p53 expression as shown in Fig. 3*E*. They did not increase luciferase activity of empty pGL3-P-luc lacking any p53BS. However, they increased transcriptional activities by more than twofold (VP16) or threefold (DXR) of pGL3-P-luc reporter vector containing either DR4-p53BS or DR5-p53BS (Fig. 3*D*). The potencies of their effects on transactivation of pP-DR4/p53BS-luc or pP-DR5/p53BS-luc correlated to their abilities to increase p53 expression. Together, these results suggest that DR4-p53BS is functionally as good as DR5-p53BS to mediate p53-induced gene transactivation.

The Intronic DR4-p53BS Is Required for Driving p53-Mediated Transactivation of DR4 Promoter. The aforementioned results clearly indicate that p53 protein binds to the intronic DR4-p53BS in cells and confers p53 responsiveness when located upstream of a promoter (*i.e.,* SV40) in a promoter-containing reporter vector (*i.e.,* pGL3-P-luc). We next wanted to know whether this p53BS also could mediate p53-dependent transcriptional activity of its own gene promoter when located at a natural position relative to the promoter in its genomic locus. To do this, we amplified a 1030-bp DR4 genomic DNA fragment consisting of the 497-bp promoter region, 371-bp exon 1, and a 262-bp fragment of the first intron harboring the DR4-p53BS or an identical 976-bp DR4 genomic DNA fragment lacking 54 bp with the DR4-p53BS. By cloning these fragments into the pGL3-B-luc vector, we generated luciferase reporter constructs with and without DR4-p53BS, which were named pB-DR4/p53BS-luc and pB-DR4/p53BS(−)-luc, respectively (Fig. 4*A*). When each of the aforementioned reporter plasmids together with the expression vector carrying no p53, wild-type p53, or mutant *p53* gene were cotransfected into H1299 cells, we found that wild-type p53 but not mutant p53 increased transcriptional activities of pB-DR4/p53B-luc by >42-fold. In contrast, p53 completely lost its ability to increase transcrip-

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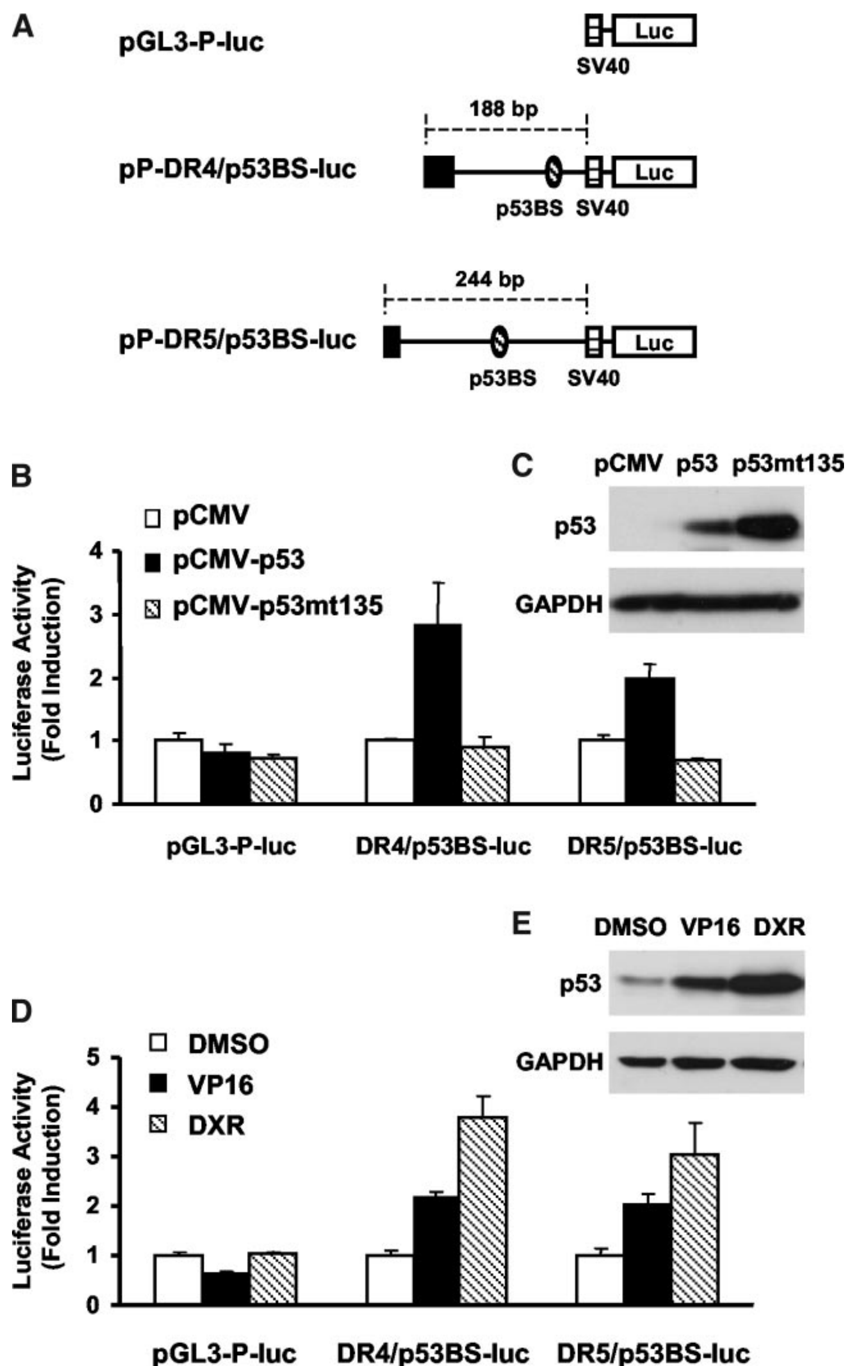

Fig. 3. Diagram illustrating cloning of DNA fragments containing death receptor 4 (*DR4*)-p53 binding site (*p53BS*) and death receptor 5 (*DR5*)-p53BS, respectively, into pGL3-promoter luciferase vector (*pGL3-P-luc*; *A*) and comparison of the activities of these reporter constructs in response to p53 overexpression (*B* and *C*) or treatment with DNA-damaging agents (*D* and *E*). *A,* the DNA fragments containing DR4-p53BS and DR5-p53BS, respectively, were PCR amplified and cloned into pGL3-P-luc reported vector upstream of the SV40 promoter. Black boxes represent partial exon 1 sequences. *B,* the indicated reporter vector, together with pCMV, pCMV-p53, or pCMV-p53mt135 and pCH110 β-galactosidase (β-gal) expression plasmid, was cotransfected into H1299 cells using the FuGene 6 transfection reagent. After 24 h, cells were harvested and subjected to a luciferase activity assay. *C,* H1299 cells transfected with pCMV, pCMV-p53, and pCMV-p53mt135 (same amounts of plasmids used in *B*), respectively. After 24 h, cells were harvested for preparation of whole cell protein lysates and subsequent Western blot analysis. *D,* MCF-7 cells transfected with the indicated reporter vector and β-gal expression plasmid using the FuGene 6 transfection reagent for 16 h were treated with 10 μM etoposide (*VP16*) or 1 μM doxorubicin (*DXR*). After 8 h, the cells were harvested and subjected to the luciferase activity assay. *E,* MCF-7 cells treated with 10 μM VP16 or 1 μM DXR for 8 h were harvested for preparation of whole cell protein lysates, which then were subjected to Western blot analysis. Each column represents the mean ± SD of triplicate treatments.

tional activity of the reporter vector with deleted DR4-p53BS [pB-DR4/p53BS(−)-luc] (Fig. 4*B*). This result clearly indicates that the intronic DR4-p53BS is essential for p53-mediated transactivation of *DR4* gene. Furthermore, we conducted transient transfection with each of the aforementioned reporter plasmids in MCF-7 cells and then treated them with VP16 or DXR. As shown in Fig. 4*C*, VP16 and DXR increased luciferase activity of pB-DR4/p53BS-luc as they did in cells transfected with the p53-luc reporter construct containing 14 repeats of consensus p53BS. However, they did not demonstrate the ability to increase luciferase activity in cells transfected with pB-DR4/p53BS(−)-luc as similar to their effects on transactivation of pGL3-B-luc. This result shows that VP16- or DXR-induced transactivation of *DR4* gene promoter requires the existence of the intronic DR4-p53BS.

Discussion

p53-dependent up-regulation of DR4 expression has been demonstrated previously by our group (10). However, the mechanism underlying p53-dependent regulation of DR4 is unknown. In the present study, we provide several lines of evidence supporting our hypothesis that p53 directly regulates transcription of *DR4* gene via an intronic p53BS. First, we identified a novel, putative p53BS in the first intron of *DR4* gene, and this p53BS is almost identical to those found in the first introns of DR5 and DcR1 in terms of their sequences and locations. Second, p53 overexpression or treatment with DNA-damaging agents enhanced the transcriptional activity of a luciferase reporter construct carrying the DR4-p53BS upstream of the SV40 promoter, indicating that this intronic p53BS is a potent enhancer of

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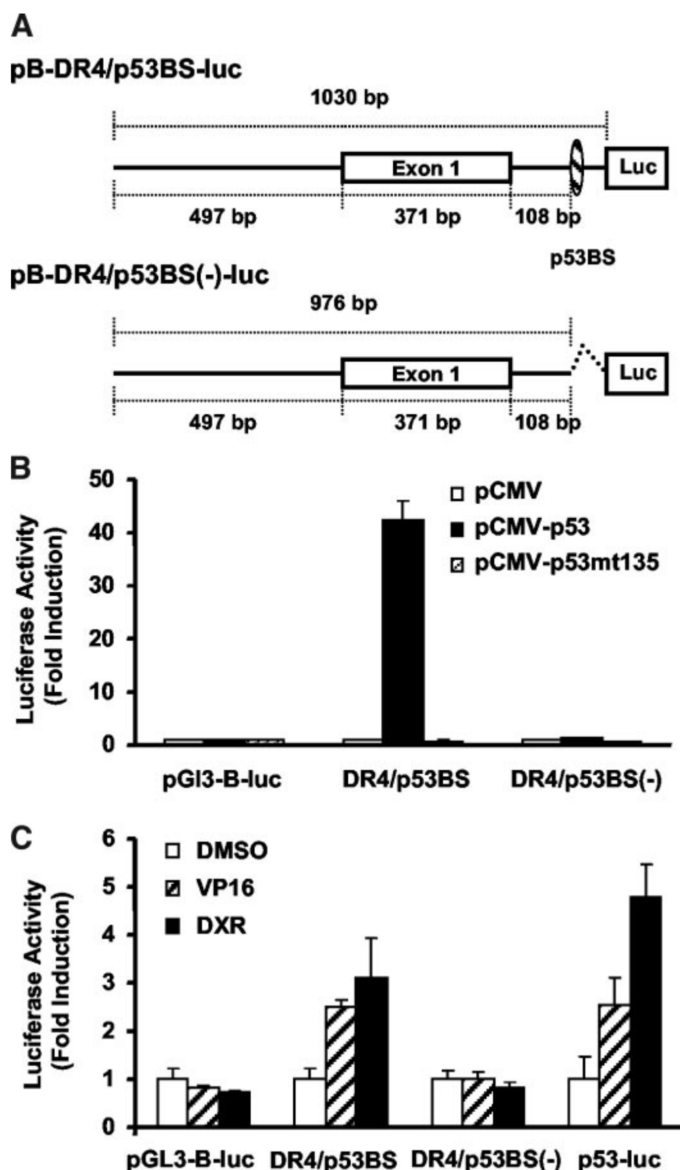

Fig. 4. Death receptor 4 (*DR4*)-p53 binding site (*p53BS*) is required for p53-dependent transactivation of DR4 promoter. *A,* diagram illustrating generation of a reporter vector that harbors a piece of natural DR4 genomic sequence with DR4 promoter region, exon 1, and partial intron 1 containing the DR4-p53BS (*pB-DR4/p53BS-luc*) and a corresponding construct lacking DR4-p53BS [*pB-DR4/p53BS(−)-luc*]. These DR4 genomic fragments were PCR amplified and cloned into a pGL-3 basic luciferase vector as described in "Materials and Methods." *B,* the indicated reporter vector, together with pCMV, pCMV-p53, or pCMV-p53mt135 and pCH110 β-galactosidase (β-gal) expression plasmid, was cotransfected into H1299 cells using the FuGene 6 transfection reagent. After 24 h, cells were harvested and subjected to luciferase activity assay. *C,* MCF-7 cells transfected with the indicated reporter vector and β-gal expression plasmid using the FuGene 6 transfection reagent for 16 h were treated with 10 μM etoposide (*VP16*) or 1 μM doxorubicin (*DXR*). After 8 h, the cells were harvested and subjected to the luciferase activity assay. Each column represents the mean ± SD of triplicate treatments.

p53-dependent transactivation. Third, the reporter construct carrying the fragment consisting of the endogenous promoter region, exon 1, and partial intron 1 region with DR4-p53BS of *DR4* gene exhibited increased transcriptional activity upon p53 overexpression or DNA-damaging insult. However, its corresponding construct just lacking DR4-p53BS did not show any response to the same stimuli. These results further indicate that DR4-p53BS is a functional enhancer element essential for p53-mediated transactivation of the *DR4* gene. Lastly, ChIP assay was able to detect DNA fragment containing DR4-p53BS from DNA/protein complex precipitated only with p53-specific antibody in Ad5-CMV-hp53-infected p53-null cell lines, indicating that DR4-p53BS binds to p53 in intact cells upon p53 activation.

The *DR4* gene is highly homologous to *DR5*, *DcR1*, and *DcR2* genes, and they map together to a tight cluster on human chromosome 8p21–22, suggesting that these genes have evolved from a common ancestral gene (19, 20). The current work and other previous studies (9, 18) have demonstrated that three (*i.e.,* DR4, DR5, and DcR1) of the four TRAIL receptors can be regulated by p53 through similar intronic p53BSs. We also have identified a similar p53BS in the first intron of *DcR2* gene, and the characterization of this p53BS is under way. Interestingly, these p53BSs have similar locations and high homology (≥90%), indicating that these p53BSs are well conserved during evolution. Therefore, it is not surprising that *DR4* gene, like other TRAIL receptors, is a p53 target gene as demonstrated in this study. These data also suggest that the four TRAIL receptors can be regulated by the same mechanism (*e.g.,* p53).

Study on tissue-specific expression of p53 target genes has suggested a key role for DR5 in p53-dependent apoptosis, particularly in spleen and small intestine *in vivo* (24). It also has been demonstrated that p53-dependent up-regulation of DR5 is required for chemosensitization of colon cancer cells to TRAIL-induced apoptosis (25). Compared with DR5, the significance of DR4 in regulation of p53-mediated apoptosis is completely unknown. Kim *et al.* (26) reported that DR4 level, but not DR5, DcR1, or DcR2 levels, correlated with TRAIL sensitivity. Ozoren *et al.* (27) have shown that homozygous deletion of *DR4* gene in a nasopharyngeal cancer cell line led to TRAIL resistance. In agreement with this study, Aza-Blanc *et al.* (28) recently reported that a small interfering RNA targeting DR5 was ineffective at blocking TRAIL-induced apoptosis, whereas small interfering RNA-mediated removal of DR4 alone conferred strong protection from TRAIL-mediated cell killing. These results clearly indicate that DR4 plays a critical role in mediating TRAIL-induced apoptosis. Coincidently, p53 overexpression was reported to augment TRAIL-induced apoptosis (13, 14, 29). More importantly, silence of p53 by a p53 small interfering RNA strongly inhibited TRAIL-induced apoptosis, indicating that p53 is required for TRAIL-induced apoptosis in some cell lines (28). Therefore, our current finding that DR4 is a p53 target gene will increase our understanding of p53-mediated apoptosis and p53-dependent enhancement of TRAIL-induced apoptosis.

In summary, we provide compelling evidence demonstrating that DR4 is a p53 target gene, which is regulated by p53 through an intronic p53BS. As the first proapoptotic TRAIL receptor identified, its roles in p53-dependent apoptosis and p53-dependent enhancement of TRAIL-induced apoptosis have not yet been elucidated.

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p53 Upregulates Death Receptor 4 Expression through an Intronic p53 Binding Site

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