Two Functionally Divergent p53-responsive Elements in the Rat Bradykinin B2 Receptor Promoter*

Jessica Marks, Zubaida Saifudeen‡, Susana Dipp, and Samir S. El-Dahr§

From the Department of Pediatrics, Section of Pediatric Nephrology, Tulane University Health Sciences Center, New Orleans, Louisiana 70112

Although p53 is known to have dual functions as a transcriptional activator and repressor, there has not been an example where both p53-activating and -repressing elements reside within one target promoter. Previous work from this laboratory defined two different p53 response elements, termed P1 and P2, located at nucleotide positions −70 and −707, respectively, in the rat bradykinin B2 receptor promoter. In this study, through manipulation of the DNA sequence and context, we demonstrate opposing roles for P1 and P2 as transcriptional activator and repressor, respectively. Deletion of P1 abrogates p53-mediated activation. P1 maintains its role as an activator upon relocation to the P2 site and activates transcription from a heterologous promoter construct. Thus, P1 is a bona fide positive p53-response element by contrast, deletion of P2 enhances P1-induced repression. P2 represses transcription when substituted for P1 or when relocated midway between P1 and P2. P2-mediated repression is sequence-dependent, because it is reversed to activation when P2 is substituted by the P1 or p53 consensus sequences. Moreover, site-directed mutagenesis that converts P2 to a higher affinity p53-binding site results in transcriptional activation rather than repression. Surprisingly, P2 strongly activates a heterologous promoter. Thus, P2-mediated transcriptional repression is both sequence- and context-dependent. Investigations into the mechanisms of P2-mediated repression indicate that it is trichostatin-insensitive and unaffected by CBP or mutation of the minimal repression C-terminal domain of p53. However, gel shift assays suggest that p53 competes with other transcriptional activators for binding to overlapping binding sequences within the P2 element. In conclusion, this study provides a rare example of a transcription factor having two divergent functional effects that are sequence- and context-dependent. The interplay of P1 and P2 may be important in the regulation of bradykinin B2 receptor gene expression in response to inflammatory stress and during development.

The tumor suppressor protein, p53, is a sequence-specific DNA-binding protein (1). Classic p53-target genes are involved in regulation of the cell cycle (e.g. p21Waf-1/Cip-1, proliferating cell nuclear antigen, Reprimo, Gadd45, 14-3-3, p22PRG1, epidermal growth factor receptor) and programmed cell death or apoptosis (e.g. Bax, Fas, p53AIP1, Pldd, Noxa, Caspase-1, Apaf-1, PUMA, KILLER/DR5) (2–14). Recent studies utilizing DNA microarray technology have identified new p53 target genes potentially involved in diverse functions, such as oxidative stress (glutathione peroxidase, cystathionine-β-synthase, and superoxide dismutase), muscle differentiation (phosphoglycerate mutase), extracellular matrix production (plasminogen activator inhibitor-1, collagen type IV, α1), membrane protein trafficking (caveolin), and vascular tone regulation (endothelin-2) (15–21). In addition, studies from our laboratory have identified a group of renal function genes (bradykinin B2 receptor, angiotensin type 1 receptor, aquaporin-2, and Na,K-ATPase) as novel p53-responsive genes (22).

p53-mediated activation of transcription is largely dependent on sequence-specific DNA binding. The consensus p53-binding motif consists of two half-sites separated by 0–13 nucleotides (18, 23). Each half-site consists of the sequence RRR/C/GAT/T/A/GYYY. p53 binds the response element as a tetramer, each half-site bound to a p53 dimer (24). p53 also represses transcription of a number of genes. The mechanisms of p53-mediated repression are not well understood, because the majority of p53-repressed genes do not contain a consensus response element. Although initial studies suggested that p53 selectively represses TATA box-containing promoters, more recent studies indicated that p53 is capable of repressing TATA-less promoters as well (25). Protein-protein interactions with the basal transcription machinery have been proposed as the underlying mechanism for p53-mediated repression of these promoters. Binding to and interference with the binding or function of upstream transcriptional activators such as SP1 and AP-1 has also been shown to mediate p53-induced repression (26–29). In addition, recruitment of chromatin-modifying enzymes or modification of the acetylation status of p53 by interaction with and recruitment of histone deacetylase plays an important role in p53-mediated repression, for some (e.g. MAP-4 and α-fetoprotein (AFP)3) but not all genes (e.g. p202) (30–33). In several instances, p53 mediates promoter repression via direct DNA binding to a canonical p53-binding motif, as has been demonstrated in the AFP, p202, and survivin genes (31, 34–36). At least in one case, the AFP gene, p53-mediated repression was dependent on displacement of an adjacent over-

3 The abbreviations used are: AFP, α-fetoprotein; BdkrB2 and B2R, bradykinin type 2 receptor gene and protein, respectively; CAT, chloramphenicol acetyl transferase; EMSA, electrophoretic gel mobility shift assay; TATA, trichostatin A; CBP, cAMP response element binding protein (CREB)-binding protein; MBD, minimal repression domain of p53; CRE, cAMP-response element; CMV, cytomegalovirus; nt, nucleotide(s); HDAC, histone deacetylase.
lapping transcriptional activator, HNF-3. In cells lacking HNF-3, p53 mediates activation rather than repression of the AFP promoter (35). In another case, the MDR1 gene, p53-mediated repression was dependent on binding to a novel p53-binding motif in which the two copies of the p53-binding site are arranged in head-to-tail orientation. Replacement of the head-to-tail site with a head-to-head site converted the activity of p53 from repression to activation (37). This finding provided the initial demonstration that inherent features in the p53-response element can confer differential functional responses. More recently, a novel 21-bp DNA element, called the p53 transcriptional repressor element (p53TRE), was identified in the promoter of the placental transforming growth factor β gene (38). This sequence (GGYYDCGGAGGCTGGARTGSWR, where R is purine, Y is pyrimidine, W is adenine or thymine, S is guanine or cytosine, and D is guanine, adenine, or thymine) diverges from the consensus p53 sequence and represses p53 transactivation in a position- and promoter-independent fashion.

We recently identified the BdkrB2 gene, which encodes the bradykinin B2 receptor (B2R), as a direct transcriptional target of p53 (39). B2R is a G protein-coupled receptor that is involved in the regulation of cardiovascular and renal functions as well as inflammation. The rat BdkrB2 promoter contains two functional p53-binding sites: a P1 site located at nucleotide positions −70; and P2, located at −707 bp, relative to the transcription start site. The P1 and P2 sequences bear 80% or greater homology to the p53 consensus sequence and bind to recombinant p53 with high affinity (22, 39). Initial promoter function analysis suggested that p53 activates the BdkrB2 promoter via the P1 site and that P2 down-modulates P1 function (39). However, the potential mechanisms of such effects were not addressed. By manipulating the sequence and context (location and spacing) of the P1 and P2 sites, we investigated in this study whether p53-mediated promoter activation and repression are dictated by the sequence of the p53-response element and/or its context. In addition, we have examined some of the potential mechanisms of P2-mediated repression within the context of the BdkrB2 promoter. The results indicate that the functions of P1 and P2 elements as activator and repressor, respectively, are dependent on their intrinsic DNA sequence. Moreover, the function of P2 as a repressor is dependent on its presence within its native promoter context, p53-mediated repression of BdkrB2 via P2 is unaffected by histone acetyltransferases, histone deacetylases, and mutation of the minimal repression C-terminal domain of p53 but may involve competitive displacement of transcriptional activators.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—HeLa cells (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Invitrogen) at 37 °C in a humidified incubator with 5% CO2. Cells were plated in duplicate in 6-well plates at 4 × 105 cells/well in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum 1 day prior to transfection. Cells were co-transfected with 1.5 μg of reporter DNA with or without the p53-expression vector, pCMV-p53. A control β-galactosidase vector pSV2 (Promega, 0.5 μg of DNA/well) was cotransfected to correct for transfection efficiency. Transfections were performed using LipofectAMINE Plus Reagent (Invitrogen) according to the manufacturer’s recommendations. Four hours after transfection, fresh medium was replaced and cell extracts were prepared 48 h later using a reporter lysis reagent (Promega). CAT activity was measured by thin layer chromatography as described after normalization for protein concentrations or β-galactosidase activity (39).

**Promoter Constructs**—The original promoter-reporter construct pB-dkrB2 −1184/+55-CAT and its derivative lacking the P2 site, −1184/CAT−ΔP2, were described (39). Utilizing the above two plasmids as templates, a series of point mutations, deletion, substitution, swap, and insertion constructs were engineered using the QuickChange site-directed mutagenesis kit (Stratagene). The promoter constructs and mutagenesis primers are depicted in Table I. The mutant clone pM340P, F341D) was obtained by the same method. The −358P2/P1 construct was generated by inserting P2 at nt position −358 in the ΔP2/P1 construct. All clones were subjected to DNA sequencing. Transfections were performed in duplicate and performed at least three times. In experiments utilizing Trichostatin A (TSA, Sigma Chemicals), TSA was added concentrations of 100 μM for 18h or 100 ng/ml for 2 h.

**Electrophoretic Mobility Shift Assays**—Bacterially produced recombinant p53 (Santa Cruz Biotechnology) or tissue nuclear extracts were used as described (22). Double-stranded oligonucleotides (Operon Biotechnologies) were 5′-end-labeled with [γ-32P]ATP with T4 kinase for use as probes in EMSA. The labeled probe was incubated for 30 min at room temperature with nuclear extracts and the binding buffer (20 mM Hepes, pH 7.9, 10% glycerol, 50 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM spermidine, 1.0 μg of poly(dI-dC)). Specific competitor oligonucleotides were added in 50- to 200-fold molar excess 15 min before addition of the radioactive probe. For supershift experiments, antibodies to p53 (phospho-ser209p53 and phospho-ser15p53, Cell Signaling; lys382 acetyl p53, Upstate Biotechnologies) were added 30 min before addition of radioactive probe. The sequences of the oligonucleotides (double-stranded) used in EMSA are as follows: P1, 5′-AGGGGAGGGAGTTGGCCGAGGAGTGTAAGCA3′; P2, 5′-ACCTTCACTGTTCGCTTGCTCTCC-3′; p53-Consensus (22), 5′-AGGATGCTGTTAAGCGCATGTG-3′; NF-κB-Consensus, 5′-AGTTAGGAGGAGCTCAGGGC-3′; KLF4 (human p21 promoter), GACCCTGCCAGGCGAGTCCC-3′; CRE-Consensus, 5′-AGGTGATGACATACCGGCCG-3′; and AP-1-Consensus, 5′-CCCTTGATGACTCAGGCGGAA-3′.

**RESULTS**

**The BdkrB2-P1 Site Is a Positive p53 Response Element**—In a previous study, we established that the rat BdkrB2 promoter has two p53-binding sites located at nucleotide positions −70 (P1) and −707 (P2), respectively, relative to the transcription start site (39) (Fig. 1A). Transient transfection assays performed in HeLa cells revealed that expression of wild-type p53 dose dependently stimulates BdkrB2 promoter-driven transcription of the CAT reporter (Fig. 1B). Deletion analysis has previously localized a positive p53-response element in the DNA fragment extending from −38 to −94 bp, which encompasses the P1 site at position −70 (39). To demonstrate conclusively that the P1 site mediates p53-induced transactivation of the BdkrB2 gene, we compared the effect of exogenous p53 on the wild type BdkrB2-CAT reporter plasmid (−1184 to −55 bp, designated P2/P1) with that of a −1184/+55 BdkrB2-CAT plasmid lacking the P1 site (designated P2/ΔP1). The results showed that elimination of P1 completely abrogates p53-mediated activation (Fig. 1C). Furthermore, elimination of P1 ablates p53-mediated activation of the −635/+55-CAT promoter construct, which lacks the repressor P2 element (Fig. 1C). We have previously shown that a DNA-binding mutant p53 cannot activate BdkrB2 transcription (39). Based on these results, we conclude that p53 mediates direct activation of the BdkrB2 promoter via the P1 p53-binding site.

To further define the role of P1, we inserted one copy of the P1 sequence upstream of the TATA box in the TATA-Luciferase reporter construct (P1/TATA-Luc). As shown in Fig. 1D, p53 causes a powerful dose-dependent activation of the heterologous promoter construct. This activation is not observed in cells transfected with pCMV-empty vector alone. In addition, as shown below, P1 is capable of transactivation when substituted for P2, although the magnitude of P1-mediated activation is higher when P1 is located closer to the basal promoter region (compare P2/P1 with P1/ΔP1 in Fig. 2B). These results confirm and extend the notion that P1 is a bona fide positive p53-response element.

**The P2 Element Mediates Sequence- and Context-dependent Repression**—To determine the role of P2, we generated several mutant versions of the original P2/P1 construct, which include single or double deletions (ΔP2/P1, P2/ΔP1, and ΔP2/ΔP1), a
swap (P1/P2), a swap with deletion (ΔP2/P2 and P1/ΔP1), and insertion with substitution (P2/P2). Deletion of the P2 site (ΔP2/P1) doubles P1-mediated activation (Fig. 2, A and B). Although the P1/ΔP1 construct is responsive to p53, the P1/P2 construct is not, confirming that P2 strongly represses P1-mediated activation. However, because P2/P1 is more responsive to p53 than P1/P2, we conclude that the location of the two elements is an important determinant of P1 and P2 function. Relocation of P2 from its natural position at nt 707 to nt 358 did not affect p53-mediated responses (compare P2/P1 and 358P2/P1, Fig. 2B), suggesting that P2-mediated repression is independent of the distance (spacing) between the P1 and P2 sites. Also, the constructs in which P1 was substituted by P2 (ΔP2/P2 and P2/P2) are highly repressed by p53 as compared with ΔP2/P1 and P2/P1 (Fig. 2, A and B). Based on these findings, we conclude that the presence of P2 dictates transcriptional repression in response to p53 in the BdkrB2 promoter. Interestingly, the double deletion construct (ΔP2/ΔP1) is responsive to p53-mediated activation (about 40% of P2/P1 activity) (Fig. 2B). Because 5′-end truncation of ΔP2/ΔP1 from −1184 to −827 (by NcoI digestion) eliminates p53-mediated activation (Fig. 2B), we conclude that an additional positive p53-responsive element resides in the region between nt −1184 and −827. The function of this upstream element becomes
apparent in the absence of P2 repressor activity.

To determine if p53-mediated repression via P2 is an intrinsic property of the P2 DNA sequence, we first replaced the P2 sequence in the P2/P1 and P2/H9004 plasmids with a consensus p53 sequence (p53-CS) (AGGCATGTCTAGGCATGTCT) to yield the plasmids CS/P1 and CS/H9004 P1. Fig. 3A shows that replacement of P2 with p53-CS converts p53-induced repression to activation. The transcriptional activation by CS and P1 tended to be additive, because deletion of P1 reduces the magnitude of p53-mediated activation (p = 0.06). In addition to P2 deletion or replacement, we evaluated the effects of P2 mutagenesis (double or triple mutations) on p53-mediated repression. Unexpectedly, a mutant P2 oligoduplex probe exhibited a higher binding activity to recombinant p53 than the wild-type P2 (Fig. 3B). To determine whether P2 interactions with p53 can be demonstrated in a more physiological setting, we incubated wild-type and triple-mutant P2 with nuclear extracts derived from newborn rat kidneys (Fig. 3C). As demonstrated here and previously (39, 40), the newborn rat kidney contains higher amounts of p53 than the adult kidney (Fig. 3D). Similar to the EMSA results using recombinant p53, the binding of the mutant P2 probe to nuclear extracts was higher than that of the wild-type probe (Fig. 3C). The presence of p53 in the nuclear extracts is demonstrated by the supershift induced by the p53 antibodies (Fig. 3C). Transient transfection assays showed that the double and triple mutant P2 constructs are much more responsive to p53 than the wild-type P2/P1 construct (Fig. 3E). Collectively, P2 deletion, replacement, and mutagenesis provide firm evidence that P2-mediated repression is sequence-specific.

To examine whether P2 function as a repressor can be maintained outside the context of the BdkrB2 promoter, we inserted three tandem P2 sites upstream of the 13 p53-binding sites in the PG13-Luc construct. Transient transfection in HeLa cells revealed that the P2(x3)-PG13-Luc construct had a higher basal activity than PG13-Luc, and p53 activated both constructs equally well (data not shown). Moreover, insertion of a single P2 site upstream of a TATA-Luc minimal promoter resulted in a strong dose-dependent p53-mediated activation rather than repression (Fig. 4). Therefore, the role of P2 as a repressor is promoter-dependent. A summary of the results of P2 and P1 mutagenesis is presented in Table II.

Mutagenesis of the Minimal Repression Domain (p53MRD) of p53 Does Not Affect P2-mediated Repression—The C-terminal segment of p53 contains an 8-amino acid region termed p53MRD (amino acids 339–346). Deletion of the p53MRD or mutagenesis of amino acids 340 and 341 abolishes p53-mediated repression of the thymidine kinase-CAT promoter (41). We therefore wished to determine what role, if any, the p53MRD
plays in P2-mediated repression. To this end, we compared the effects of pCMV-p53 (wild-type) or pCMV-p53 (M340P/F341D) on the transcriptional activity of P2/P1, P2/P2, and P2/P1. Because wild-type p53 is known to repress viral promoters (5), we first tested the effect of p53MRD mutagenesis on pSV-lacZ promoter activity. As expected, wild-type p53 inhibited the expression of β-galactosidase in a dose-dependent manner; in contrast, p53 mutant MRD had no significant effect (Fig. 5A). In addition, mutations of the p53 MRD did not affect p53-mediated repression of the P2/P1, P2/P2, or P2/P1 constructs (Fig. 5B). Interestingly, the mutant p53M340/F341D retained its ability to activate transcription albeit at a lower efficiency than the wild-type p53 (Fig. 5B).

**Role of Histone Acetylase and Deacetylase**—Recruitment of HDACs to the promoter by p53 has been shown to mediate p53 repression of certain promoters and could account for P2-mediated repression (33). Also, HDACs may theoretically deacetylate p53. To test these possibilities, we first treated HeLa cells with trichostatin A (TSA), a selective HDAC inhibitor, at doses shown previously to reverse p53-mediated transcriptional repression. We then performed transient transfection assays with the p53 and the promoter-reporter constructs. As shown in Fig. 6A, treatment with TSA has no appreciable effect on P2-mediated repression of the P2/P1 and P2/P1 constructs, indicating that p53-mediated repression is probably exerted by a mechanism that is independent of HDAC activity. In the absence of p53, TSA stimulated slightly the basal activity of the promoterless CAT construct (Fig. 6A).

We previously reported that p53-mediated activation of the P2/P1 construct is further enhanced in the presence of co-transfected CBP-expressing vector (39). We surmised that if P2 interferes with P1 function by competition for the available intracellular pool of CBP/p300, constructs lacking P2 but having P1 should respond more vigorously to CBP/p300 (and p53) than those with an intact P2 site. CBP enhanced p53-mediated activation by 40% above that of p53 alone. However, the magnitude of activation of P2/P1 and P2/P1 was not different (Fig. 6B).
Differential Binding of \( p53 \) to \( P1 \) and \( P2 \)—Differences in the binding affinity of the \( P1 \) and \( P2 \) elements to \( p53 \) might account, at least partly, for their differential roles as activator and repressor, respectively. We therefore compared the binding activity of recombinant \( p53 \) to \( P1 \) and \( P2 \) using EMSA (Fig. 7A). The protein-DNA complex is efficiently competed by unlabeled \( P1 \) or \( P2 \) in a dose-dependent manner. As judged from the amount of competitor oligoduplex required to displace \( p53 \) binding, the relative affinity of \( p53 \) for \( P1 \) is \(-30\% \) higher than \( P2 \) (Fig. 7B). We next tested the binding of radiolabeled \( P1 \) and \( P2 \) to newborn rat kidney nuclear extracts. The protein-DNA complexes produced using \( P1 \) were more abundant and migrated more slowly than those of \( P2 \). These qualitative and quantitative differences prompted further investigations into whether other nuclear DNA-binding proteins share with \( p53 \) the ability to bind to \( P2 \). A transcription factor search revealed that the \( P2 \) sequence has putative overlapping binding sites for \( NF-\kappa B \) and Kruppel-like factor-4 (formerly known as GKL2) (Fig. 8A). Competitive EMSA utilizing the \( P2 \) probe and unlabeled \( p53-CS \), \( P1 \), \( NF-\kappa B \), \( AP-1 \), \( KLF-4 \), and \( cAMP \)-response element (CRE) oligoduplexes were performed (Fig. 8B). The \( P2 \) probe produced three specific DNA-protein complexes (C1–C3). C1 is competed completely by unlabeled \( p53 \) CS. Unlabeled \( P1 \) also competes well against \( P2 \) for binding. Interestingly, C1 is reduced equally well by unlabeled KLF-4 and to a lesser extent by CRE and Jun/AP-1 but not by \( NF-\kappa B \) oligoduplex. C2 is competed only weakly by \( p53 \) CS, \( P1 \), and other oligoduplexes. C3 is completely eliminated by unlabeled KLF-4 oligoduplex and decreased by unlabeled \( NF-\kappa B \) and \( AP-1 \) duplexes. These findings indicate that \( P2 \) is a potential binding site for multiple transcription factors, including \( p53 \), \( NF-\kappa B \), and KLF-4 and possibly others, and suggest that \( p53 \) competes with \( NF-\kappa B \) and KLF-4 for binding to the \( P2 \) sequence.

**DISCUSSION**

The present study demonstrates that the transcription factor \( p53 \) can simultaneously activate and repress a target gene via sequence-specific binding to two different cis-response elements. In the case of the rat \( BdkrB2 \) promoter, the end-result is transcriptional activation as the \( P1 \) element greatly overpowers the \( P2 \) repressor element. Multiple lines of evidence suggest that \( P1 \) is a bona fide positive \( p53 \)-response element: 1) the minimal \( BdkrB2 \) promoter \((-38/+55)\) cannot respond to \( p53 \); in contrast, a \(-94/+55) \) promoter construct containing the \( P1 \) \( p53 \)-binding site \((-50 \text{ to } -70)\) is highly \( p53 \)-responsive \((22, 39)\); 2) deletion of \( P1 \) abrogates \( p53 \)-mediated activation; 3) relocation of \( P1 \) (e.g. \( P1/D1 \)) maintains \( p53 \)-mediated activation; and 4) \( P1 \) mediates activation of a heterologous minimal promoter-reporter construct. Conversely, several observations support the notion that \( P2 \) is a sequence-specific repressor element: 1) interference with \( P2 \) function by either truncation, deletion, or mutagenesis up-regulates \( p53 \)-mediated activation via \( P1 \); 2) \( P2 \) represses transcription when substituted for \( P1 \); 3) substitution of \( P2 \) by a consensus \( p53 \)-binding sequence converts the repression to activation; and 4) \( P2 \) maintains its repressor activity within the context of the \( BdkrB2 \) promoter irrespective of \( P2/P1 \) spacing. However, once taken out of its native context and placed in front of a foreign promoter, \( P2 \) behaves as an activator. Thus, the role of \( P2 \) is dictated by both its own sequence and the inherent properties of the surrounding DNA elements.

The mechanisms of \( p53 \)-mediated transcriptional repression are complex and not fully understood but include sequence
nonspecific and sequence-specific mechanisms (26–38). Non-
specific repression includes binding to and interference with
basal transcription factors, binding to an activator (e.g. SP-1 or
AP-1), or competition for a co-activator. Sequence-specific re-
pression includes direct DNA binding to a p53 response ele-
ment, recruitment of a co-repressor (e.g. mSin3/HDAC complex)
and competition for binding with other transcription factors
with overlapping binding sites. Our results indicate that TSA,
a selective HDAC inhibitor, had no effect on P2-mediated re-
pression. Thus, a direct role for HDAC in histone or p53
deaetylation does not appear to be a mechanism. This is not
surprising, however, because not all p53-repressed genes are
TSA-sensitive. We also tested the possibility that P1 and P2
compete for a common co-activator, such as CBP/p300. How-
ever, forced CBP expression in the absence of P2 had no addi-
tional influence on p53-mediated activation. This finding sug-
gests that competition for CBP is not a contributing factor.

We further considered whether p53-mediated repression via
P2 is determined by structural elements in the p53 protein
itself. Hong et al. (41) have identified a minimal repression
domain (p53MRD) that inhibits p53-mediated transcriptional
activation. The MRD maps to an 8-amino acid (339–346) seg-
ment of the C-terminal of native p53. The function of this
domain is selective for p53, because it did not affect VP16-
methylation activation. Also, mutations of two amino acids,
M340P and F341D, were sufficient to prevent p53-mediated
repression of the thymidine kinase promoter. We introduced
p53M340P/F341D into HeLa cells and determined its effects on
transcription from the P2/P1 construct and its mutant deriva-
tives. We reasoned that, if the p53MRD contributes to P2-
mediated repression, then the mutant p53 should not have
such an effect. The results showed that P2-mediated repression
is maintained in the presence of p53M340P/F341D. Interest-
ingly, the mutant p53 construct exhibited a weaker transcrip-
tional activity than native p53, despite similar transfection
efficiency. These results suggest that p53-mediated repression
via P2 is not dependent on the p53MRD. Although the MRD is
the site of E6 binding by the papilloma viral protein and HeLa

**Fig. 7.** Binding characteristics of P1 and P2 to p53. A, electrophoretic mobility shift assay (EMSA). Double-stranded 32P-labeled P1
(5'-GGAGGTGCCCAGGAGAGTGA-3') and P2 (5'-ACTCTTGGCTGTCTTTCC-3') oligoduplexes (40,000 cpm/reaction) were incubated with 500
ng of recombinant p53. Increasing amounts of cold competitor oligoduplex (50- to 150-fold) were added to the reaction mixture. B, densitometric
analysis of the band intensities in A. C, EMSA utilizing radiolabeled p53 consensus sequence (CS), P1 or P2 (20,000 cpm/reaction), incubated with
5 μg of nuclear proteins extracted from newborn rat kidneys. Increasing amounts of cold competitor oligoduplex (50- to 100-fold) were added to the
reaction mixture. Arrows point to specific DNA-protein complexes.
cells express the E6 protein, impaired E6-p53 interactions cannot explain the loss of repression mediated by mutant p53MRD. p53MRD-mediated repression may be more relevant to non-sequence-specific repression seen with overexpression of p53 than to sequence-specific repression. For example, p53-mediated repression of the survivin gene is not affected by mutation or deletion within the C-terminal domain, including the MRD (36). In the case of the P2 element, our results clearly indicate the three DNA-protein complexes. See “Experimental Procedures” for the sequences of the oligonucleotides.

An important finding of this study is that P2 mediates repression of BdkrB2 promoter regardless of whether or not P1 is present and irrespective of the spacing between P1 and P2. Also, because replacement of P2 with P1 or the p53 consensus sequence strongly activates transcription and mutagenesis of P2 increases its p53-binding activity and enhances transcription, we suggest that P2-mediated repression of the survivin gene as B2R levels (22, 40). Further, p53-deficient pups on C57BL6 background manifest ectopic B2R expression in the kidney (22). Thus, p53 may regulate the developmental spatio-temporal expression of the BdkrB2 gene. It is not clear, however, how the interplay between P1 and P2 modulates these processes. Whereas P1 is evolutionally conserved between rat, mouse, and human, P2 is not. This raises the intriguing possibility that the BdkrB2 gene has acquired enhanced responsiveness to p53 during evolution. Perhaps P2 serves to restrict P1-mediated activation of the BdkrB2 gene during terminal differentiation.

In summary, the rat BdkrB2 gene represents a rare example of a mammalian gene that is reciprocally regulated by a single transcription factor via sequence-specific DNA binding. Although promoters with more than one functional p53-binding site have been described (e.g. p21 and cyclin G) (42), the presence of two functionally divergent p53-binding elements within the same promoter is a novel finding. This should offer a useful system to investigate how p53 activates or represses gene transcription in the context of the endogenous promoter. More importantly, this system may allow us to determine whether the divergent p53-mediated transcriptional responses are stimulus-dependent (e.g. DNA damage versus development).

Acknowledgments—We thank Drs. R. Kwok, G. Morris, and B. Vogelstein for generous contribution of plasmids and William Baricos for critical review of the manuscript.

REFERENCES
1. Kern, S. E., Kinzler, K. W., Bruskin, A., Jarosz, D., Friedman, P., Pries, C., and Vogelstein, B. (1991) Science 252, 1728–1731
2. el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) Cell 75, 817–825
3. Morris, G. F., Bischoff, J. R., and Mathews, M. B. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 895–899
4. Jackson, P., Ridgway, P., Rayner, J., Noble, J., and Braithwaite, A. (1994) Mol. Cell. Biol. 14, 6785–6794
5. Shivakumar, C. V., Brown, D. R., Deb, S., and Deb, S. P. (1995) Mol. Cell. Biol. 15, 6785–6794
6. Otsu, H., Nomoto, J., Murasawa, H., Oda, E., Inazawa, J., Tanaka, N., and Taniguchi, T. (2000) J. Biol. Chem. 275, 22627–22630
7. Schaffer, H., Trauzold, A., Sebens, T., Deppert, W., Fuchs, U. R., and Schmidt, W. E. (1998) Oncogene 16, 2479–2487
8. Deb, S. P., Munoz, R. M., Brown, D. R., Sahler, M. A., and Deb, S. (1994) Oncogene 9, 1341–1349
9. Mitry, R. R., Sarruf, C. E., Wu, C. G., Pignatti, M., and Habib, N. A. (1997) Lab. Invest. 77, 369–378
10. Munsch, D., Watanabe-Fukunaga, R., Bourdon, J. C., Nagata, S., May, E., Yonish-Rouach, E., and Reisdorf, P. (2000) J. Biol. Chem. 275, 3867–3872
Transcriptional Regulation of Kinin B2 Receptor Gene by p53

11. Lin, Y., Ma, W., and Benchimol, S. (2000) Nat. Genet. 26, 122–127
12. Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. (2000) Science 288, 1053–1058
13. Nakano, K., and Wouda, K. H. (2001) Mol. Cell 7, 683–694
14. Takeda, R., and El-Deiry, W. S. (2000) Oncogene 19, 1728–1743
15. Cui, X. S., and Donehower, L. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 13099–13104
16. Lo, P. K., Chen, J. Y., Tang, P. P., Lin, J., Lin, C. H., Su, L. T., Wu, C. H., Chen, T. L., Yang, Y., and Wang, F. F. (2001) J. Biol. Chem. 276, 37186–37193
17. Wang, L., Wu, Q., Qiu, P., Mirza, A., McGuirk, M., Kirschmeier, P., Greene, J. R., Wang, Y., Pickett, C. B., and Liu, S. (2001) J. Biol. Chem. 276, 43604–43610
18. Zhao, R., Goh, K., Murphy, M., Yin, Y., Notterman, D., Hoffman, W. H., Tom, E., Mack, D. H., and Levine, A. J. (2000) Genes Dev. 14, 45–49
19. El-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W., and Vogelstein, B. (1992) Nat. Genet. 1, 45–49
20. Xu, D., Wang, Q., Gruber, A., Bjorkholm, M., Chen, Z., Zaid, A., Selivanova, G., Peterson, C., Wiman, K. G., and Pisa, P. (2000) Oncogene 19, 5123–5133
21. Webster, N. J., Resnik, J. L., Reichart, D. B., Strauss, B., Haas, M., and Seely, B. L. (1996) Cancer Res. 56, 2781–2788
22. Barlev, N. A., Liu, L., Chehab, N. H., Mansfield, K., Harris, K. G., Halazonetis, T. D., and Berger, S. L. (2001) Mol. Cell 7, 1243–1254
23. Oden, S. K., Lee, K. C., Werner-Dallies, K., Stratton, S. A., Aronow, B., and Barton, M. C. (2001) J. Biol. Chem. 276, 42057–42062
24. Espinosa, J. M., and Emerson, B. M. (2001) Mol. Cell 8, 57–69
25. Murphy, M., Ahn, J., Walker, K. K., Hoffman, W. H., Evans, R. M., Levine, A. J., and George, D. L. (1999) Genes Dev. 13, 2490–2501
26. DiPace, S., Xu, D., Wang, Q., Gruber, A., Bjorkholm, M., Chen, Z., Zaid, A., Selivanova, G., Peterson, C., Wiman, K. G., and Pisa, P. (2000) Oncogene 19, 5123–5133
27. Webster, N. J., Resnik, J. L., Reichart, D. B., Strauss, B., Haas, M., and Seely, B. L. (1996) Cancer Res. 56, 2781–2788
28. Barlev, N. A., Liu, L., Chehab, N. H., Mansfield, K., Harris, K. G., Halazonetis, T. D., and Berger, S. L. (2001) Mol. Cell 7, 1243–1254
29. Oden, S. K., Lee, K. C., Werner-Dallies, K., Stratton, S. A., Aronow, B., and Barton, M. C. (2001) J. Biol. Chem. 276, 42057–42062
30. Espinosa, J. M., and Emerson, B. M. (2001) Mol. Cell 8, 57–69
31. Murphy, M., Ahn, J., Walker, K. K., Hoffman, W. H., Evans, R. M., Levine, A. J., and George, D. L. (1999) Genes Dev. 13, 2490–2501
32. D'Souza, S., Xin, H., Walter, S., and Choueby, D. (2001) J. Biol. Chem. 276, 298–305
33. Lee, K. C., Crowe, A. J., and Barton, M. C. (1999) Mol. Cell. Biol. 19, 1279–1288
34. Hoffmann, W. H., Biade, S., Zifroni, J. T., Chen, J., and Murphy, M. (2002) J. Biol. Chem. 277, 3247–3257
35. Johnson, R. A., Ince, T. A., and Scott, K. W. (2001) J. Biol. Chem. 276, 27716–27720
36. Tan, M., Wang, Y., Guan, K., and Sun, Y. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 169–114
37. Saifudeen, Z., Du, H., Dipp, S., and El-Dahr, S. S. (2000) J. Biol. Chem. 275, 15557–15562
38. Saifudeen, Z., Marks, J., Du, H., and El-Dahr, S. S. (2002) Am. J. Physiol. Renal Physiol. 283, F727–F733
39. Hong, T. M., Chen, J. J., Peck, K., Yang, P. C., and Wu, C. W. (2001) J. Biol. Chem. 276, 1510–1515
40. Zauberman, A., Lupo, A., and Oren, M. (1995) Oncogene 10, 2361–2366