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Advances in Brief

Identification of a p53-dependent Negative Response Element in the *bcl-2* Gene[1]

Toshiyuki Miyashita, Masayoshi Harigai, Motoi Hanada, and John C. Reed[2]

La Jolla Cancer Research Foundation, Cancer Research Center, La Jolla, California 92037

Abstract

Recently, we have shown that the p53 tumor suppressor gene product can inhibit expression of the *bcl-2* gene. In this report, we explored the molecular basis for p53-mediated down-regulation of *bcl-2* gene expression using a cotransfection approach involving p53 expression plasmids and chloramphenicol acetyltransferase (CAT) reporter gene constructs containing regions from the *bcl-2* gene. When transfected into a p53-deficient human lung cancer cell line H358, reporter gene constructs containing only the promoter region of *bcl-2* and upstream sequences were not suppressed by p53. Inclusion of *bcl-2* gene sequences corresponding to the 5′ untranslated region in *bcl-2*/CAT constructs, however, resulted in p53-dependent down-regulation. A 195-base pair segment from the *bcl-2* gene 5′ untranslated region was found to be capable of conferring p53-dependent repression on a heterologous expression plasmid containing CAT under the control of an SV40 immediate early-region promoter. This p53-negative response element functioned in an orientation-independent manner when placed either upstream or downstream of the SV40-CAT transcription unit. The results demonstrate the existence of a negative response element in the *bcl-2* gene through which p53 may either directly or indirectly transcriptionally down-regulate expression of this gene involved in the regulation of programmed cell death.

Introduction

Loss of p53 tumor suppressor gene function occurs frequently in human tumors, suggesting that this event plays a critical role in the development or progression of many types of cancer (1). The mechanisms responsible for p53 inactivation include gene deletion, somatic and germline point mutations, overexpression of mdm-2 (a dominant inhibitor of p53), and inactivation by proteins encoded by DNA tumor viruses, such as the E6 protein of human papilloma viruses and SV40 large T antigen which bind to and neutralize the function of the p53 protein through various mechanisms (2–4).

Though p53 may have additional biochemical activities (5), this DNA-binding protein has been shown to function as a transcriptional regulator in a variety of circumstances (6–8). For example, p53 can mediate transcriptional activation of genes containing at least two copies of a 10-bp sequence motif that constitutes a specific binding site for this protein (2, 6–8). Conversely, p53 has been shown to down-regulate a variety of promoters that lack this p53 response element, including those for MDR-1, c-*fos*, β-actin, and hsc70 (9, 10). In at least some cases, this down-regulation may occur via interactions of p53 with the TATA-binding factor involved in transcription initiation in genes that contain a TATAA box (11, 12).

Recently, we obtained evidence that the p53 tumor suppressor gene product can down-regulate *bcl-2* gene expression both *in vitro* and *in vivo* (13). Specifically, expression of a temperature-sensitive p53 mutant in the p53-deficient murine myeloblastic leukemia cell line M1 was shown to result in reductions in *bcl-2* mRNA and protein levels upon shift to permissive temperature. In addition, immunohistochemical and immunoblot analysis of Bcl-2 protein levels in p53-deficient transgenic mice ("p53 knock-outs") revealed elevated levels of Bcl-2 protein in several tissues including spleen, thymus, lymph nodes, and prostate. Bcl-2 protein levels, however, were not increased in all tissues in p53 knock-out mice, and the usual tissue and differentiation stage-specific patterns of *bcl-2* gene expression were preserved. These findings suggest that while p53 may be able to influence the relative output of the *bcl-2* gene, it does not supercede other regulatory mechanisms that control the expression of this gene whose encoded protein is a blocker of programmed cell death (also known as "apoptosis"; reviewed in Ref. 14).

To begin to delineate the mechanisms by which p53 can inhibit *bcl-2* gene expression, we explored the effects of p53 on reporter gene constructs containing potential regulatory regions from the human *bcl-2* gene. The results reveal that a PNRE[3] is located in a portion of the *bcl-2* gene corresponding to the 5′ UTR.

Materials and Methods

Plasmid Constructions. Fig. 1 (*top*) depicts the structure of the *bcl-2* gene promoter region and the various reporter gene constructs used for this report. Restriction fragments corresponding to the *bcl-2* gene promoter and 5′ UTR were excised from the *bcl-2* genomic clone p18–21H (15) and subcloned into the *Hind*III site immediately upstream of CAT in either the promoterless plasmid pUCSV0-CAT (TM438–2, TM460–1, and MYH38–1) or pUCSV3-CAT (TM378–3), which contains a SV40 early-region promoter 5′ of the *Hind*III site (16). The plasmids pTM438–2, pTM460–1, pMYH-38–1, and pTM378–3 contain the following *bcl-2* gene fragments, respectively: 3.3 kbp *Hind*III/*Sac*I; 3.7 kbp *Bam*HI to the *bcl-2* ORF; 2.4 kbp *Bam*HI/*Sac*I; and 1.3 kbp *Nae*I to the ORF. The plasmids pTM438–2 and pMYH38–1 were created by blunt-end ligation, after filling in the ends of both inserts and vectors using a combination of Klenow fragment and T4 DNA polymerase. To replace the *bcl-2* ORF with CAT for construction of pTM460–1 and pTM378–3, p18–21H plasmid DNA was subjected to PCR amplification using Pfu polymerase under the manufacturer's recommended conditions (Stratagene, Inc.) and a forward primer (5′-CCTGCTTTCACTCAGTGTGTACAGG-3′) which contains an internal *Dra*III site (*underlined*) and a reverse primer (5′-GACGTAGATCAAGCTTCCTTCCAGAGGAA-3′) representing a mutagenic primer designed to place a *Hind*III site (*underlined*) precisely at the position normally occupied by the ATG of the *bcl-2* ORF. This resulting PCR product was digested with *Dra*III and *Hind*III and subcloned into *Dra*III and *Hind*III sites of pUC18 into which a 1.6-kbp *Nae*I/*Nae*I fragment from p18–21H had been subcloned, creating the plasmid pTM358–2 (not shown). The *Eco*RI site in pTM358–2 was converted to *Hind*III using an adaptor, and this plasmid was digested with *Hind*III to liberate the 1.3-kbp fragment for pTM378–3. For pTM460–1, pTM358–2 was digested with *Bgl*II and *Bam*HI, and the larger band was gel purified and ligated with a 3.45-kbp *Bam*HI/*Bgl*II fragment from p18–21H, thus creating a 3.7-kbp *bcl-2* gene segment from the *Bam*HI site shown in Fig. 1 to the ORF in pUC18. This gene segment was then excised with *Bam*HI and *Hind*III and subcloned into the *Bgl*II and *Hind*III sites of pUCSV0-CAT, which are located −10 and 0 bp upstream of CAT in this promoterless plasmid (16).

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[2] To whom requests for reprints should be addressed, at La Jolla Cancer Research Foundation, Cancer Research Center, 10901 N. Torrey Pines Rd., La Jolla, CA 92037.

[3] The abbreviations used are: PNRE, p53-negative response element; UTR, untranslated region; ORF, open reading frame; PCR, polymerase chain reaction; β-gal, β-galactosidase; kbp, kilobase pairs; bp, base pairs; Pfu, plaque-forming unit.

In addition, a DNA fragment corresponding to the human *bcl-2* gene region, −279 to −85 bp which contains a PNRE, was amplified from p18–21H using Pfu DNA polymerase and specific forward (5′-GCGAAGCTTGTAGACT-GATATTAAC-3′) and reverse (5′-GCGAAGCTTATAATCCAGCTATTTT-3′) primers that contained a *Hind*III site (*underlined*). The amplification product was subcloned into the unique *Hind*III site of pUCSV3CAT or pUCSV0CAT in either the correct ("sense," S) or reversed ("antisense," AS) orientation, thus creating the plasmids SV40-PNRE-S-CAT, SV40-PNRE-AS-CAT, and PNRE-S-CAT. To subclone the PNRE upstream or downstream of the SV40-CAT transcriptional unit in pUCSV3-CAT, the unique *Hind*III site was destroyed by blunting with Klenow and self-ligation. A *Bam*HI site (downstream of CAT) and a *Bgl*II site (upstream of SV40 promotor) were converted to *Hind*III sites by use of appropriate linkers, and the PNRE fragment was subcloned into those new *Hind*III sites, generating the plasmids SV40-CAT-PNRE and PNRE-SV40-CAT, respectively. Proper construction of all plasmids was confirmed by DNA sequencing.

Transfections. H358 cells (17) were plated in 6-well plates, and transfections were performed when the cells reached 70% confluency. Each well received 3 μg of p53 expression plasmid (CMV-p53$_{wt}$ or CMV-p53$_{179}$; Ref. 8), 3 μg of reporter gene plasmid, 1 μg of pCMVβ-Gal (18), and 30 μg of Lipofectin (GIBCO BRL) in 2.5 ml of HL-1 medium (Ventrex) for 16 h. Cells were scraped into Hanks' balanced salt solution 48 h after transfection, washed two times with Hanks' balanced salt solution, and resuspended in 100 μl of ice-cold 0.25 M Tris (pH 7.8), subjected to three freeze-thaw cycles, and centrifuged at 16,000 × *g* for 5 min to obtain supernatants.

Reporter Gene Assays. CAT assays were performed using 30 μl of cell lysates and 0.2 μCi [*acetyl*-^{3}H]coenzyme A (4.48 Ci/mmol; NEN), and the results were normalized relative to β-gal activity (19). For both CAT and β-gal assays, serial dilutions of standard enzymes were always included to verify that results were within the linear phase of the reactions, and the volumes of cell lysates were adjusted as necessary. Normalization for protein concentration instead of β-galactosidase yielded comparable results (not shown).

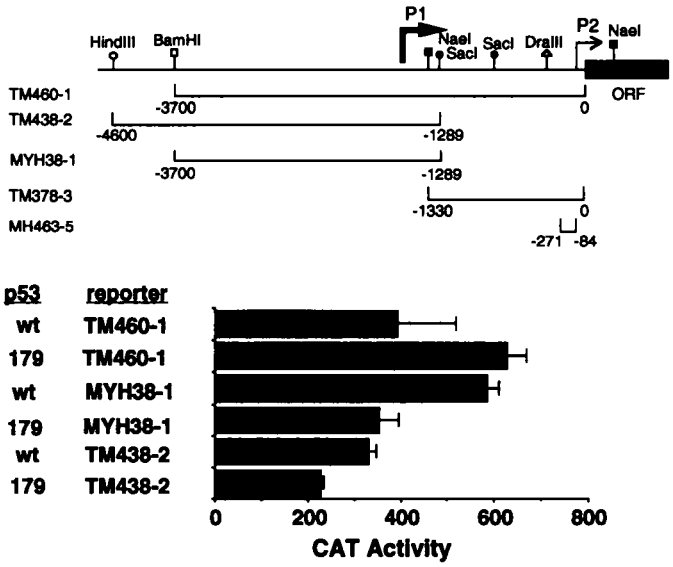

Fig. 1. A p53-negative response element is located in the 5′ UTR of the human *bcl-2* gene. The structure of various *bcl-2*/CAT constructs is depicted (*top*), showing the regions of the *bcl-2* gene included in either the promoterless CAT reporter gene plasmid pUCSV0-CAT (pTM438–2, pTM460–1, and pMYH38–1) or the SV40 immediate-early region containing plasmid pUCSV3-CAT (pTM378–3 and pMH463–5). Locations of selected restriction sites are indicated, as well as the positions of the major (P1) and minor (P2) transcription start sites in the *bcl-2* gene relative to the ORF (15, 20). CAT reporter gene plasmids were cotransfected into H358 cells with either CMV-p53$_{wt}$ or CMV-p53$_{179}$ plasmids. CAT activity (cpm) normalized for β-galactosidase production via pCMV-β-gal (absorbance, 405 nm) was measured 2 days later (*bottom*). Data represent mean for three determinations; *bars*, SD.

Results

The structure of the *bcl-2* gene is complex, and its promoter regions are relatively poorly characterized thus far. The prinicipal region (P1) where transcripts initiate has been mapped to an area containing several GC boxes (Sp1-binding sites) located ~1.7 kbp upstream of the ORF (Fig. 1). This configuration leads to the production of a long 5′ UTR in *bcl-2* mRNAs. A second potential promoter (P2) is located ~80 bp upstream of the coding region, but fewer than 5% of all transcripts initiate from this second promoter region (20).

To explore the mechanism by which p53 down-regulates *bcl-2* gene expression, CAT reporter gene constructs were prepared that contained segments of the *bcl-2* gene. These *bcl-2*/CAT constructs were cotransfected with expression plasmids encoding either normal p53 or a mutant p53(179) protein that lacks transcriptional activity (8) into H358 cells, a human lung cancer line that contains no p53 protein due to homozygous *p53* gene deletions (17). In an effort to emulate the configuration of the *bcl-2* gene, initially a *bcl-2*/CAT plasmid was prepared (pTM460–1) in which a CAT reporter gene was subcloned into a genomic clone containing ~3.7 kbp of human *bcl-2* gene sequences upstream of the translation start site at position +1 of the *bcl-2* ORF, thus essentially substituting CAT for the *bcl-2* ORF (Fig. 1). Comparisons of the relative levels of CAT activity generated from this reporter gene construct when cotransfected with either p53$_{wt}$ or p53$_{179}$ expression plasmids revealed that the transcriptionally active p53$_{wt}$ protein resulted in a modest but reproducible decrease in CAT activity relative to the mutant, transcriptionally incompetent p53$_{179}$ protein in p53-deficient H358 human lung carcinoma cells (Fig. 1, *bottom*). In an effort to map the location of sequences within the *bcl-2* gene that were required for this p53-dependent down-regulation, two additional reporter gene constructs were then prepared that fused CAT to *bcl-2* gene fragments derived from the region upstream and including the P1 start site, thus deleting the 5′ UTR sequences (pTM-438–2 and pMYH-38–1; Fig. 1). Comparisons of relative levels of CAT activity generated from these constructs when cotransfected with either p53$_{wt}$ or p53$_{179}$ expression plasmids revealed that this region upstream of the *bcl-2* major transcription start site failed to mediate p53-dependent down-regulation in the absence of the 5′ UTR of the *bcl-2* gene (Fig. 1). In fact, p53$_{wt}$ paradoxically induced an increase in the activity of these *bcl-2*/CAT reporter gene plasmids relative to the negative control p53$_{179}$, suggesting that removal of the 5′ UTR unmasked a potential p53-positive response element location within the *bcl-2* gene.

Since the results shown in Fig. 1 implied that a PNRE was located in the region of the *bcl-2* gene somewhere between the major transcription start site and the translation initiation site, we next prepared a construct (pTM-378–3) where the *bcl-2* 5′ UTR was subcloned between a SV40 early-region promoter/enhancer and CAT in the plasmid pUCSV3-CAT (Ref. 16), thus substituting SV40 for the *bcl-2* promoter (Fig. 1). RNA blotting assays and reverse-transcription PCR mapping studies confirmed that the transcripts generated from pTM-378–3 contained the *bcl-2* 5′ UTR fused to CAT with transcripts initiating from the SV40 promoter (not shown). Relative to the SV40-CAT plasmid (pUCSV3-CAT) that lacked *bcl-2* sequences, the reporter gene plasmid containing the *bcl-2* 5′ UTR generated lower basal levels of CAT activity when transfected alone (not shown) or in combination with the p53$_{179}$ expression plasmid or other control plasmids (Fig. 2*A*), suggesting the presence of a p53-independent inhibitory element within this region of the *bcl-2* gene. However, when cotransfected with the p53$_{wt}$ expression plasmid, a further decrease in CAT activity was detected, implying that a PNRE resided in this region of the *bcl-2* gene.

Recently, a negative regulatory element has been identified in the *bcl-2* gene between −271 bp and −84 bp relative to the translation initiation start site (21). Therefore, to test the effects of p53 on this segment of the *bcl-2* gene and to emulate the normal configuration of

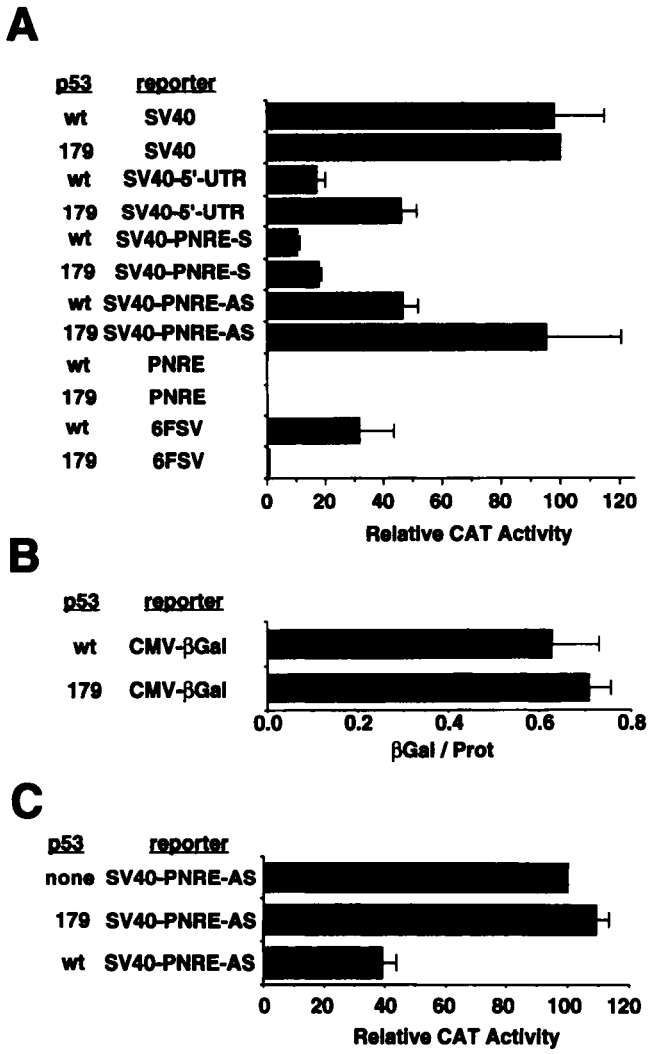

Fig. 2. The −279- to −85-bp region of the human *bcl-2* gene contains two types of negative regulatory elements. CAT reporter gene plasmids were cotransfected into H358 cells with either CMV-$p53_{wt}$, CMV-$p53_{179}$, or pRc/CMV (Invitrogen, Inc.) plasmid DNAs along with pCMV-β-gal. Relative CAT activity was measured 2 days later and normalized for either β-gal activity (*A* and *C*) or protein content (*B*). Reporter gene plasmids included: pUCSV3-CAT lacking *bcl-2* gene sequences; SV40-5′-UTR (pTM378-3) containing the ~1.3-kbp *bcl-2* 5′ UTR subcloned in the sense orientation between the SV40 promoter and CAT reporter gene in pUCSV3-CAT; SV40-PNRE-S-CAT and SV40-PNRE-AS-CAT containing the −279 to −85-bp region of *bcl-2* subcloned between the SV40 promoter and CAT in either sense (*S*) or antisense (*AS*) orientation in pUCSV3-CAT; a promoterless PNRE-CAT construct where the *bcl-2* (−279/−85-bp) fragment was subcloned upstream of CAT in pUCSV0-CAT; and p6FSV-CAT, a plasmid containing six copies of a p53-positive response element (4). In some cases, data are normalized relative to results obtained for the SV40-CAT plasmid pUCSV3-CAT lacking *bcl-2* sequences when cotransfected with CMV-$p53_{179}$ (*A*) or when cotransfected with the negative control plasmid pRc/CMV (C). *B*, data are expressed as arbitrary $A_{405\ nm}$ units of β-gal normalized for total cellular protein content. All data represent mean ($n \geq 3$); *bars*, SD.

the *bcl-2* gene, a *bcl-2* (−279/−85) fragment was subcloned between an SV40 early-region promoter and CAT in both sense and antisense orientations, and the ability of this plasmid to produce *bcl-2*/CAT fusion transcripts when transfected into cells was confirmed by RNA blotting and reverse transcription-PCR mapping (not shown). Cotransfection experiments were then performed using pCMV-$p53_{wt}$ and pCMV-$p53_{179}$ plasmids.

When inserted between the SV40 early-region promoter and the CAT gene in its usual (sense) orientation, the −279- to −85-bp fragment markedly suppressed the basal production of CAT activity (Fig. 2*A*). This result implies that the *bcl-2* 5′ UTR contains both positive and negative regulatory elements that are independent of p53 and that a potent p53-independent repressor element lies within the −279- to −85-bp region. However, when the SV40-*bcl-2*(−279/−85-bp)-CAT plasmid was cotransfected with active (wild-type) p53, a further ~50% reduction in CAT activity was consistently seen, relative to cotransfections performed with the $p53_{179}$ plasmid (Fig. 2*A*). Interestingly, when subcloned in reversed (antisense) orientation between the SV40 promoter and CAT in pUCSV3-CAT, the −279- to −85-bp fragment had essentially no inhibitory effect on the SV40-CAT reporter gene unless cotransfected with expression plasmids encoding wild-type p53 (Fig. 2*A*). Compared to the nonfunctional $p53_{179}$ mutant, wild-type p53 inhibited by about one-half (58 ± 18; $n = 5$) the expression of the SV40-CAT construct, which contained the −279- to −85-bp fragment in antisense orientation. Cotransfection experiments using variable amounts of pCMV-$p53_{wt}$ and pCMV-$p53_{179}$ plasmid DNAs with a fixed amount of the *bcl-2*-PNRE-AS-CAT reporter plasmid DNA (total DNA amount was adjusted with pRc/CMV plasmid DNA to hold constant) revealed a concentration-dependent inhibition of CAT activity by $p53_{wt}$ which plateaued at ~50% in H358 cells, at least under the transfection conditions used here (data not shown). Taken together, the results derived from comparisons of the −279/−85-bp *bcl-2* fragment when inserted in sense and antisense orientations into the SV40-CAT reporter plasmid suggest that this region of the *bcl-2* gene contains both a p53-independent negative regulatory element that functions in an orientation-dependent manner and a p53-dependent negative regulatory element that functions in an orientation-independent fashion.

Cotransfection of the wild-type p53-encoding plasmid with an SV40-CAT plasmid that lacked *bcl-2* sequences demonstrated that p53 does not inhibit the SV40 promoter in H358 cells and confirmed the dependence on the *bcl-2* PNRE for down-regulation of CAT activity by p53 (Fig. 2*A*). Expression of the pCMV-β-gal plasmid used to normalize for transfection efficiency was also not inhibited by $p53_{wt}$ (Fig. 2*B*). Studies of a CAT reporter gene construct that contained the *bcl-2* PNRE without an SV40 promoter showed that the −279- to −85-bp region of *bcl-2* has no transcriptional activity of its own in H358 cells, despite the presence of CAAT and TATAA boxes in this DNA fragment (20). When expression plasmids producing either $p53_{wt}$ or mutant $p53_{179}$ were cotransfected with a plasmid that contains six copies of a p53-positive response element cloned upstream of a minimal promoter and CAT, p6FSV-CAT (4), increases in CAT activity were produced with the former but not the latter (Fig. 2*A*). These data obtained with the p6FSV-CAT reporter gene construct argue that production of $p53_{wt}$ does not lead to a general decrease in gene expression in H358 cells and thus demonstrate the specificity of the observed down-regulation of CAT reporter gene constructs that contained the *bcl-2* PNRE. Comparisons of the effects of a control expression plasmid pRc/CMV, which encodes no protein with pCMV-$p53_{179}$, which produces a mutant inactive form of p53, demonstrated no significant effect of the mutant p53 protein on reporter gene constructs that contained the *bcl-2* PNRE (Fig. 2*C*). Thus, while some p53 mutants can function as dominant inhibitors of wild-type p53 (6–8), the $p53_{179}$ protein did not appear to influence the expression of these *bcl-2*/CAT reporter gene constructs, at least in H358 cells which have homozygous *p53* gene deletions (17).

Because the SV40-PNRE-AS-CAT construct used for these experiments produced *bcl-2*-CAT fusion transcripts, it was possible that p53-mediated down-regulation could occur at the translational level, as well as through other posttranscriptional mechanisms, such as by blocking transcript elongation. Therefore, we prepared reporter gene constructs where the *bcl-2* PNRE was located outside of the SV40-CAT transcriptional unit at either the 5′ or 3′ sides. Regardless of whether the *bcl-2* PNRE was located upstream or downstream of the SV40-CAT transcriptional unit, p53-mediated inhibition was ob-

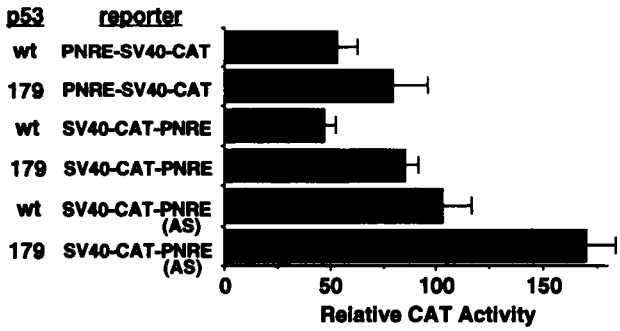

Fig. 3. The *bcl-2* PNRE functions irrespective of position in pUCSV3-CAT. Cotransfections and CAT assays were performed as described for Fig. 1. The reporter gene plasmids included: PNRE-SV40-CAT and SV40-CAT-PNRE, where the *bcl-2* (−279/−85 bp) region was positioned either upstream or downstream of the SV40-CAT transcriptional unit in the same 5′→3′ orientation; and SV40-CAT-PNRE (*AS*), where the PNRE was subcloned in reverse orientation (antisense) downstream of the SV40-CAT transcriptional unit.

served based on comparisons of CAT activity obtained in cotransfection experiments using plasmids encoding functional $p53_{wt}$ *versus* mutant inactive $p53_{179}$ (Fig. 3) or other negative control plasmids (data not shown). Furthermore, the *bcl-2* PNRE appeared to function irrespective of orientation (sense *versus* antisense) when placed 3′ of the SV40-CAT transcriptional unit in these reporter plasmids. Taken together, these findings suggest, therefore, that the p53-negative response element mapped to the −279/−85-bp region of the *bcl-2* gene operates at the transcriptional level.

Discussion

In the majority of non-Hodgkin's B-cell lymphomas, t(14;18) chromosomal translocations bring the *bcl-2* gene at 18q21 into juxtaposition with the immunoglobulin heavy-chain locus at 14q32, resulting in transcriptional deregulation of the *bcl-2* gene (22, 23). In addition to lymphomas with t(14;18) translocations, high levels of Bcl-2 protein, aberrant patterns of Bcl-2 protein production, or both have been observed in a variety of solid tumors (24–27). In contrast to lymphomas, however, no evidence for gross alterations in *bcl-2* gene structure has been obtained for these other types of cancer, suggesting that alternative mechanisms for dysregulation of *bcl-2* gene expression may exist in human malignancies.

In this report, we have used *bcl-2*/CAT reporter gene plasmids and cotransfection assays to map the approximate location of a p53-negative response element in the *bcl-2* gene. When taken together with other recent data showing that p53 down-regulates Bcl-2 protein levels in cell lines *in vitro* and in several tissues *in vivo* (13), the findings imply that loss of p53-mediated repression of *bcl-2* gene expression may account, at least in part, for the frequent abnormalities in Bcl-2 protein production seen in tumors that do not harbor chromosomal translocations involving the *bcl-2* gene.

The PNRE identified in the *bcl-2* gene appeared to have the characteristics of a transcriptional "silencer" in that it mediated p53-dependent repression in an orientation- and position-independent manner. Although this 195-bp segment from the 5′ UTR of the human *bcl-2* gene was able to confer p53-dependent repression on a heterologous promoter, it remains to be determined whether the repression of *bcl-2* by p53 occurs directly or through another transcriptional regulator whose production is regulated by p53. Moreover, we cannot exclude the possibility of additional elements within the *bcl-2* gene that may play a role in p53-mediated suppression of *bcl-2* gene transcription. Examination of the DNA sequence of the *bcl-2* gene revealed an absence of the consensus-positive response element for wild-type p53 (2); however, p53 is known to down-regulate a variety of gene promoters that lack this p53-response element (9, 10).

The region of the *bcl-2* gene that comprises a p53-negative response element also appears to contain an additional p53-independent inhibitory element that is both orientation- and position-dependent, as described previously (21). Specifically, when the −279- to −85-bp region of *bcl-2* was placed between an SV40-promoter and CAT in correct (sense) orientation, potent suppression of CAT gene expression was observed in p53-deficient H358 cells (Fig. 2*A*) as well as other p53-null tumor cell lines (data not shown). However, when positioned upstream or downstream of the SV40 promoter and CAT gene or between the SV40 promoter and CAT in reversed (antisense) orientation, no repression was observed in the absence of p53. Further mapping of the specific sequences required for function of these p53-dependent and -independent elements and identification of the factors that presumably bind there is necessary before the relative importance and possible interplay of these inhibitory elements can be determined. It could be, for example, that loss of both p53 and the unknown factor that represses via the *bcl-2* −279- to −85-bp segment in an orientation- and position-dependent fashion would be required to achieve complete deregulation of *bcl-2* gene expression in tumors. Analysis of p53 knock-out mice, however, has suggested that loss of p53 alone is sufficient to result in elevated *bcl-2* gene expression in at least some tissues *in vivo* (13).

The p53 protein has been shown previously to bind to and interfere with the activity of the TATA-binding factor (11, 12). Though the region of the *bcl-2* gene to which the PNRE maps contains a TATAA sequence, this element was not used as a site for transcription initiation in our constructs, particularly since the PNRE was functional in reversed (antisense) orientation. Furthermore, preliminary experiments indicate that deletion of the TATAA box from the 195-bp fragment (−287/−85-bp) does not diminish its ability to confer p53-dependent repression on heterologous gene constructions.[4] Thus, it seems unlikely that the interactions of p53 with a TATA-binding factor account for the inhibition of *bcl-2* gene expression. In this regard, it should be noted that the major *bcl-2* promoter (P1) lacks a TATAA box and instead has a "housekeeping" gene configuration involving multiple Sp-1 binding sites and several transcription initiation sites (20).

Although p53 inhibited expression of *bcl-2*/CAT constructs containing the PNRE by only ~50% in H358 cells, preliminary experiments suggest that the magnitude of the p53 response in cotransfection assays varies among tumor cell lines and thus may be tissue-specific.[4] These tissue-specific differences notwithstanding, recent reports demonstrating an inverse correlation between Bcl-2 immunostaining in breast cancer specimens and positive p53 immunostaining, which is often an indicator of mutant p53 proteins and loss of wild-type p53 function (28), as well as transfection studies showing that expression of a mutant p53 protein in some breast cancer cell lines results in diminished *bcl-2* gene expression (29), indicate that regulation of the *bcl-2* gene by p53 may be complex and subject to the influences of other factors. It may be noteworthy in this regard that $p53_{wt}$ up-regulated *bcl-2*/CAT reporter gene constructs that contained the *bcl-2* (P1) promoter region in the absence of the *bcl-2* 5′-UTR (Fig. 1, *bottom*). Therefore, further studies are required to delineate the relative importance of p53 and the PNRE located between −279 and −85 bp in the overall regulation of *bcl-2* gene expression in normal tissues and various types of cancers. Nevertheless, the evidence presented here and elsewhere (13) indicating that p53 can down-regulate *bcl-2* gene expression in cultured cells *in vitro* and in

[4] Unpublished observations.

some tissues *in vivo* may have important implications for the mechanisms by which p53 regulates apoptosis and sensitivity to chemotherapeutic drugs and radiation (30–34).

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