Published in final edited form as:

Gene. 2007 December 1; 404(1-2): 110–116.

Identification of a Novel Bcl-2 Promoter Region that Counteracts in a p53-Dependent Manner the Inhibitory P2 Region

Sebastian Bredow, Daniel E. Juri, Karen Cardon, and Yohannes Tesfaigzi*

Respiratory Immunology and Asthma Program, Lovelace Respiratory Research Institute, 2425 Ridgecrest Dr. SE, Albuquerque, NM 87108, USA

Abstract

Expression of the anti-apoptotic proto-oncogene *bcl-2* is negatively affected by the pro-apoptotic p53. To understand the regulation of *bcl-2* expression by p53, we studied the *bcl-2* promoter regions individually and in the context of the full-length promoter. While the P1 promoter displayed the highest p53-independent activity, the P2 promoter activity was suppressed in p53-sufficient cancer cell lines. In addition, P2 activity was higher in primary airway epithelial cells from *p53*^{-/-} mice compared to those from *p53*^{+/+} mice. Chromatin immunoprecipitation assays confirmed that p53 interacts within a 140 bp sequence of P2 that contained the CCAAT- and TATA-elements. However, when P1 and P2 are linked in one construct, P2 suppressed P1 activity independent of p53. A potential novel promoter with a p53-dependent activity was identified located between P1 and P2, and was designated M. In the context of the full-length *bcl-2* promoter, M counteracted in a p53-dependent manner the suppressive activity of P2 on P1. Collectively, these data suggest that P1 promoter is the main driving force for transcribing the *bcl-2* gene and P1 activity is modulated by M and P2 in a p53-dependent and -independent manner. These findings may have implications for therapies that are geared towards inhibiting *bcl-2* gene expression and inducing cell death.

Keywords

bcl-2 gene transcription; promoter activity; negative regulatory element; lung; airway epithelium

1. INTRODUCTION

The proto-oncogene product Bcl-2 inhibits apoptosis (Vaux et al., 1988; Hockenbery et al., 1990). Our previous studies show that following inflammatory responses Bcl-2 protein expression is crucial in regulating the number of cells in airway epithelia (Tesfaigzi et al., 2000; Harris et al., 2005; Tesfaigzi, 2006). It is estimated that approximately 50% of human cancers have increased levels of Bcl-2 (Reed, 1998), and *bcl-2*-specific antisense phosphothioate oligonucleotides are used in cancer therapies for non-Hodgkin's lymphoma to inhibit *bcl-2* gene expression and induce apoptosis of tumor cells (Webb et al., 1997; Manion and Hockenbery, 2003). However, a better understanding of *bcl-2* gene regulation is needed to improve successful therapy (Reed, 1999). In various tumor cells, high levels of p53 are often associated with low levels of Bcl-2 and *vice versa* (Harn et al., 1996; Lee et al., 1996; Hoehner et al., 1997). Bcl-2 protein levels in p53-deficient mice are elevated in the spleen, thymus,

*To whom correspondence should be addressed: Yohannes Tesfaigzi, Lovelace Respiratory Research Institute, 2425 Ridgecrest Dr. SE, Albuquerque, NM 87108, Tel: (505) 348-9495, Fax: (505) 348-8567, E-mail: ytesfaig@lrrr.org

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

lymph nodes, and prostate compared to wild-type mice (Miyashita et al., 1994b), suggesting that p53 has an inhibitory effect on *bcl-2* gene transcription (Budhram-Mahadeo et al., 1999). p53 is a DNA-binding transcription factor that can activate gene expression by directly binding to specific DNA sequences (el-Deiry et al., 1992) or can function through protein-protein interactions with other transcription factors to alter gene expression.

Regulation of the *bcl-2* gene has been investigated in cells from predominantly hematopoietic lineage (Miyashita et al., 1994b; Chen and Boxer, 1995; Wilson et al., 1996; Catz and Johnson, 2001; Wu et al., 2001). These studies have identified two promoters that mediate tissue- and developmental-specific *bcl-2* gene transcription (Young and Korsmeyer, 1993). The TATA-less and GC-rich P1 promoter is considered to be dominant with a transcriptional start site identified 1386-1423 bp upstream of the open reading frame (Seto et al., 1988). The P2 promoter is located 1.3 kilobases (kb) downstream of P1, contains both CCAAT and TATA sequences and minor initiation sites at positions -31 and -58 (Seto et al., 1988). A p53-dependent negative regulatory element (NRE) is believed to repress expression in lung cancer cells via a 195 bp region within the P2 promoter (Miyashita et al., 1994a). No p53-binding site is located in this region, but studies in hematopoietic cells have demonstrated that p53 may exert its effect through interaction at the TATA element (Wu et al., 2001) or through association with the CCAAT-box activator NF-Y (Imbriano et al., 2005).

The roles of the P1 and P2 *bcl-2* promoter regions in airway epithelial cells have not been investigated and the role of the region located between P1 and P2 that we designate as M has not been evaluated. Therefore, the purpose of the present studies was to investigate whether P1 and P2 regions have roles in airway epithelial cells similar to those described in hematopoietic cells and to examine the function of the M region. We present evidence that p53 affects transcriptional activity of the P2 promoter in pulmonary epithelial cells but that in the context of the full-length promoter P2 suppresses P1 promoter activity in a p53-independent manner. Furthermore, we show that the M region is a potentially independent promoter that is regulated by p53 and within the context of the full-length promoter in a p53-dependent manner counteracts the suppressive activity of P2.

2. Materials and methods

2.1. Cell culture

Two p53-sufficient human epithelial lung cancer-derived cell lines, A549 and Calu-3, and two p53-deficient cell lines, H1299 (carcinoma, non-small cell lung cancer), and SaOS-2 (bone osteosarcoma) were maintained in RPMI medium (Invitrogen; Carlsbad, CA) supplemented with 10% (or 15% in case of SaOS-2) (v/v) fetal bovine serum (HyClone; Logan, UT), 100 U/ml penicillin and 10 µg/ml streptomycin (all from Invitrogen) in a humidified 5% CO₂ atmosphere at 37°C. Primary airway epithelial cells were isolated from the tracheas of *p53*^{+/+} and *p53*^{-/-} mice on C57Bl/6 background as described (You et al., 2002) and placed in culture on 0.4 µm-pore-size Transwell membranes (Costar; Pleasanton, CA).

2.2. Bcl-2 promoter constructs

A schematic of all constructs is shown in Fig. 1. Constructs containing either the full-length promoter (LB322: -3934 to -8) or the P1 promoter (LB124: -3934 to -1287) in Bluescript (Stratagene, La Jolla, CA) were kindly provided by Dr. Linda M. Boxer (Stanford University School of Medicine, Stanford, CA). The restriction endonucleases *Bam*HI and *Hind*III (New England Biolabs; Beverly, MA) were used to subclone the full-length promoter into the promoterless vector, pGL3-Basic (Promega, Madison, WI) that contains the reporter gene firefly luciferase to yield plasmid P1-M-P2 (GenBank accession no. **EU119400**). The P1 promoter construct was obtained by digesting LB124 with *Spe*I and *Hind*III and inserting the

P1 promoter region into *NheI* (which has *SpeI*-compatible overhangs)/*HindIII* restricted pGL3-Basic. The P2 promoter construct was obtained by subcloning the P2 (-755 to start) region from LB322 into pGL3-Basic following restriction with *SacI* and *HindIII*. Competitor plasmid P2-TA was obtained by cloning the PCR-amplified P2 product into the TA cloning vector pCR2.1 (Invitrogen; Carlsbad, CA). The region separating P1 from P2, designated as M, was obtained by deleting P2 and P1 from P1-M-P2 by restriction with *BglII/HindIII* and *Apal/KpnI* respectively. Blunt ends were generated using the Klenow fragment of *E. coli* DNA polymerase I and ligated to construct the M plasmid in pGL3-Basic. To obtain construct P1-M, the P2 promoter was excised from P1-M-P2 using *BglII/HindIII*, and the resulting gel-purified product treated with Klenow fragment of *E. coli* DNA polymerase I to generate blunt ends, and religated. The P1 promoter was excised from P1-M-P2 with *Apal/KpnI*. Following gel purification, blunt ends were generated using the Klenow fragment of *E. coli* DNA polymerase I; religation yielded plasmid M-P2. Construct P1-x-P2, with a deleted M region was generated by linearizing plasmid P1 with *HindIII* followed by inserting of the gel-purified P2 promoter, which was obtained after excision from plasmid P2-TA with *HindIII*. To generate a deletion construct in which 140 bp containing the CAAT- and TATA-box were deleted the P2 promoter region was amplified by PCR using P1-M-P2 as template with forward primer CTCTTGAGATCTCCGGTTGGGAT and reverse primer GTTCCGCGTGATTGAA. The PCR product was digested with *HindIII/NdeI* and cloned into P1-M-P2 to replace P2 and generate P1-M-ΔP2. The proper construction of all plasmids was confirmed by DNA sequencing using four different dyes to identify the A, C, G, and T extension reactions. Sequencing was carried out sequenced using the dideoxynucleotide chain termination method at the facilities provided by the Keck-UNM Genomics Resource Center.

2.3. Transient transfection assays

All transfections were carried out using 24 well-plates (USA Scientific; Ocala, FL) with the exception of transfections for chromatin immunoprecipitation (ChIP) assays, which were performed in 10 cm-plates using a total of 6 μg DNA. Briefly, cells were < 30% confluent when transfected; all transfections were carried out in duplicate. Plasmids (0.2 μg *bcl-2* promoter construct, 0.1 μg *Renilla* luciferase-expressing reporter vector pRL-TK (Promega; Madison, WI), and 0.2 μg competitor when needed) were co-transfected with FuGENE 6 transfection reagent (Roche Diagnostics; Indianapolis, IN) at a FuGENE:DNA ratio of 3:1. Cells were lysed 48h later, and promoter activity analyzed in a Luminoskan Ascent luminometer (Thermo Electron; Milford, MA) using the Dual Luciferase® Reporter Assay System (Promega). The firefly luciferase values were normalized to those of *Renilla* luciferase, the expression of which was under the control of the human thymidine kinase promoter to control for well-to-well variation and transfection efficiency. Unless otherwise stated, all transfections were repeated at least three times.

2.4. Chromatin immunoprecipitation assays

ChIP assays were performed using a Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology; Lake Placid, NY) with the following modifications. Forty-eight h after transfection with constructs P1-M-P2, P2, or P1-M-ΔP2, A549 cells in 10 cm-plates were treated with formaldehyde (final concentration of 1%) for 10 min at room temperature (RT). Cross-linking was stopped by adding glycine to a final concentration of 0.125 M and incubating for 5 min at RT. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) containing protease inhibitors (Complete Mini; Roche), scraped and resuspended in 1ml SDS Lysis buffer (50 mM Tris pH 8.1, 10 mM EDTA, 1% SDS, plus protease inhibitors) per 2×10^7 cells. After 10 min on ice, the lysates were sonicated with 15 pulses of 10 sec using a Branson Sonifier 450 and then cleared by centrifugation. The supernatants were diluted 10-fold in ChIP Dilution Buffer (16.7 mM Tris pH 8.1, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, with protease inhibitors), precleared with Protein A agarose beads that were saturated

with 1 mg/ml bovine serum albumin and 0.4 mg/ml salmon sperm DNA, and then incubated overnight at 4°C with either no or 2 µg of a mouse monoclonal p53-specific antibody (sc-98; Santa Cruz, CA) on a rotating wheel. Antibody-bound chromatin was immunoprecipitated with Protein A agarose beads, the beads were collected by centrifugation and then sequentially washed in Low Salt (10 mM Tris pH 8.1, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100), High Salt (same as Low Salt except 500 mM NaCl), LiCl Immune Complex Wash buffer (10 mM Tris pH 8.1, 250 mM LiCl, 1 mM EDTA, 1% IGEPAL-CA630, 1% Na-deoxycholate), and twice in TE (10 mM Tris pH 8.0, 1 mM EDTA). Chromatin was eluted from the beads by two 15 min incubations with freshly prepared Elution buffer (0.1 M NaHCO₃, 1% SDS). Following the addition of 5 M NaCl, cross-links of the combined eluates as well as input/starting material (1% diluted supernatant corresponding to 10,000 cells) were reversed by heating to 65°C for 5h. DNA was purified by first incubating all samples with 20 µg Proteinase K (Roche) for 90 min at 45°C, followed by the addition of potassium acetate-solution (60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml H₂O). Samples were vigorously mixed, incubated on ice for 5 min, and centrifuged at maximum speed for 5 min at 4°C. The supernatants were transferred to fresh microfuge tubes containing 0.6 ml iso-propanol. DNA precipitates were recovered after 3 min of centrifugation at RT. Following a wash in 70% ethanol, DNA was resuspended in 50 µl TE buffer. Conditions for PCR using 2 µl of DNA have previously been described (Bredow et al., 2000); position of primers (sense: 5'-AAGTGTTCGCGTGATTGA, anti-sense: 5'-TTTCTTTATGTTTTTGGCGTCTTC) and amplified fragment sizes are indicated in Figure 1.

2.5. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) of three independent transfections, each done in duplicates. Student's unpaired t-tests (two-tailed) were used to analyze differences in promoter activities of two constructs. Analysis of variance (ANOVA) was performed on data for more than two groups followed by the Tukey-Kramer post hoc test when statistically different. *P*-values of <0.05 were considered statistically significant.

3. Results

3.1. p53 interacts with the P2 promoter region and suppresses P1 promoter activity

The effect of p53 on *bcl-2* promoter activity was investigated in epithelial cell lines that either had wild-type p53 (A549, Calu-3) or were p53-deficient (SaOS-2 and H1299). Cells were transiently transfected with luciferase reporter plasmids, containing either the full-length P1-M-P2 promoter or the individual promoters, P1 or P2 (Fig. 1). The activity of all promoter constructs in H1299 cells was ~10-fold higher than the other cell lines. The P1 promoter showed the highest activity of all constructs in all cell lines irrespective of p53 status (Fig. 2A). However, P2 promoter activity was decreased to ~20% of that for P1 in p53-sufficient cells, and only to 90 and 50% in p53-deficient SaOS-2 and H1299 cells, respectively. Promoter activity for P1-M-P2 was lower than that of P1 in all cell lines.

While previous studies have analyzed the role of P2 in various cancer cell lines (Catz and Johnson, 2001; Wu et al., 2001), the effect of p53 on P2 promoter activity has not been studied in primary epithelial cells. Therefore, airway epithelial cells were isolated from three *p53*^{+/+}- or *p53*^{-/-}-mice and transfected with either the P1- or P2-promoter luciferase reporter constructs. While the P2 promoter sequence is highly conserved among mice and humans (95%), sequence homology for P1 is only ~ 50%. Thus, higher activity for human P2 than for human P1 was expected in *p53*^{-/-} mouse airway cells. Results (Fig. 2B) showed that absolute P1 activity was similar in both *p53*^{+/+}- and *p53*^{-/-}-mouse cells. As expected, compared to cells from *p53*^{+/+}- mice, activity for P2 was significantly increased in primary cells from *p53*^{-/-}-mice.

To confirm that the tumor suppressor p53 interferes with *bcl-2* promoter activity through interaction within the 195 bp NRE of the P2 promoter (Miyashita et al., 1994a), chromatin immunoprecipitation (ChIP) experiments were performed. The p53-sufficient human pulmonary epithelial cell line A549 was transiently transfected with *bcl-2* promoter constructs P1-M-P2, P2, or P1-M-ΔP2, a construct lacking the CCAAT- and TATAA-boxes (Fig. 1). Primers for the ChIP were designed to amplify construct-specific sequences but not the promoter sequences of the endogenous gene. Thus, DNA amplification was observed in transfected cells but not in non-transfected controls (Fig. 3A; lanes labeled with NT: DNA isolated from non-transfected cells; lanes labeled with i: input DNA isolated from transfected cells). Immunoprecipitation with anti-p53 specific antibodies followed by PCR resulted in amplification of construct-specific fragments in cells transfected with P1-M-P2 and P2 but not P1-M-ΔP2 (Fig. 3A; lanes labeled with +); controls were immunoprecipitations without antibody (Fig. 3A; lanes labeled with -). These findings are consistent with previous reports (Wu et al., 2001; Imbriano et al., 2005) that p53, most likely through interaction with other proteins, interferes with *bcl-2* gene expression via the NRE.

3.2. p53 blocks assembly of transcription factors on the P2 region

We wanted to test whether P2 promoter activity would be affected by co-transfecting the P2 DNA sequences by competing for binding of transcription factors. Promoter activities for P1-M-P2, P1, and P2 were determined in A549 and H1299 cells co-transfected with either P2-TA or empty TA vector as control. When compared to cells transfected with the empty-TA vector, the presence of P2-TA significantly decreased activity of P1-M-P2 and P2 constructs by approximately 23% only in p53-deficient H1299 cells, while activity of P1 was not affected in either cell line (Fig 3B). None of the luciferase activities was affected by the P2-TA in p53-sufficient A549 cells. The absolute activities for P1, P2, and P1-M-P2 were similar to those observed in Fig. 2. The observation that P2 has increased promoter activity only in p53-deficient cells suggests that transcription factors interact with the P2 promoter only in the absence of p53.

3.3. p53 and the role of the M promoter region

Our data show that compared to P1 promoter activity suppression of P1-M-P2 activity was more pronounced in p53-sufficient (~ 47% decreased activity) than in p53-deficient (7-31% decrease) cells (Fig. 2A). Furthermore, while P2 appeared to suppress the activity of P1 in p53-sufficient cells, it was not clear why P1 and P2 activities were not additive in p53-deficient cells where P2 is active. These observations led us to investigate a possible p53-dependent function of M, the region between P1 and P2. Analyses of M promoter activity in the p53-sufficient and p53-deficient cells revealed that this 530 bp-long M sequence supports expression of reporter gene independent of either P1 or P2 sequences (Fig. 4A). The activity of this potentially novel promoter was 50% of P1 in p53-sufficient cells but approximately 50% higher than P1 promoter activity in p53-deficient cells. However, M did not affect P1 promoter activity in p53-sufficient or -deficient cells in the absence of P2 (Fig. 4A). Furthermore, when M was present upstream of P2 in the absence of P1, activity of M was essentially abrogated in p53-sufficient cells, but M-P2 activity was approximately 50% higher than P2 only in p53-deficient cells (Fig. 4B) suggesting that P2 suppressed M activity in a p53-dependent manner.

To further investigate the role of M in the context of the full-length promoter we generated the construct P1-x-P2, in which M was deleted from the *bcl-2* promoter. As was reported previously (Miyashita et al., 1994a), P2 suppressed P1 activity independent of p53 status (Fig. 4C). However, compared to P1-M-P2, activity of P1-x-P2 was higher in p53-sufficient cells but was reduced in p53-deficient cells (Fig. 4C) suggesting that M in the presence of p53 plays a role in counteracting the P1-suppressing activity of P2.

4. Discussion

The present studies show that the M region, which separates the established promoters P1 and P2, has promoter activity and that it counteracts in a p53-dependent manner the suppressing activity of P2. Furthermore, we present evidence that interactions of 3 independent promoter regions affect *bcl-2* gene expression and these promoter regions fulfill different roles within the context of the full-length promoter.

4.1. P2 promoter activity is p53-dependent

Compared to P2 promoter P1 displayed the highest promoter activity irrespective of cellular p53 status, suggesting that p53 does not affect the activity of this promoter and that the P1 promoter region is the main driving force for transcribing the *bcl-2* gene. In contrast, promoter activity of P2 was strongly affected by p53, because compared to P1 activity P2 activity was reduced by ~80% in p53-sufficient but only by 10-40% in p53-deficient cell lines. The increase of P2 promoter activity in the absence of p53 was reproduced in primary airway epithelial cells from *p53*^{+/+} and *p53*^{-/-} mice. Previous studies have studied P2 promoter activity in various cancer cell lines. To our knowledge, this is the first report that analyzed P2 promoter activity in primary cells.

The ChIP assays further support the fact that p53 interacts with the P2 region where the CCAAT and TATA elements are present. Our data are consistent with previous reports showing that the P2 activity is lower when compared to P1 (between 30-50%) (Miyashita et al., 1994a), and that the inhibitory effect of p53 on *bcl-2* promoter activity is approximately 2-fold (Miyashita et al., 1994a). Our data also confirm previous reports that p53 suppresses P2 promoter activity through a 195 bp NRE via the CCAAT- or TATA-box elements that are separated by 13 bp (Miyashita et al., 1994a). Our findings suggest that in pulmonary epithelial cells p53 *in vivo* interacts within this part of the NRE suggesting that regulation of *bcl-2* gene expression is similar to hematopoietic cells.

4.2. p53 affects the assembly of transcription factors on the P2 region

The fact that P2 promoter activity was suppressed by co-transfected TA vector containing the P2 DNA sequence in p53-deficient but not p53-sufficient cells further supports the hypothesis that p53 may affect the assembly of transcription factors at the P2 promoter. It is possible that p53 forms a stable complex with transcription factor complexes on P2 to keep them inactive, and that they are not easily sequestered by P2 sequences when co-transfected as P2-TA. However, it is more likely that binding of p53 to P2 diminishes promoter activity by sterically inhibiting the binding of critical transcription factor(s) to P2, because promoter activity is not completely abolished. The finding that P1-M-P2 activity is reduced by co-transfected P2-TA suggests that P2 may be active even in the context of the whole promoter. That the P2 promoter displays a low-level activity in pulmonary epithelial cells that is reduced in the presence of p53 is consistent with a previous report (Miyashita et al., 1994a). In p53-deficient cells assembled transcription factors can be sequestered by the competing P2-TA construct leading to decreased promoter activity of both P1-M-P2 and P2. Absence of p53 may lead to the formation of intact transcription factor complexes and increased P2 activity. That in p53-sufficient cells the low activity of P2 remains low even in the presence of competing P2 sequences can be explained by p53 hindering binding of transcription factors to the P2 region.

Interestingly, although P2 promoter activity is suppressed by p53 activity of the main transcriptional promoter, P1, is significantly reduced when linked to P2 irrespective of p53-status. The mechanisms underlying this apparent discrepancy between the p53-dependent regulation of P2 as an independent promoter and p53-independent suppression of P1 within

the context of the full-length *bcl-2* promoter is unclear but suggests that P2 may function in a p53-dependent manner under certain cellular conditions.

4.3. p53 and the M promoter region

We hypothesized that p53 may affect promoter activity through interactions with regions other than P2. This assumption led to the discovery that the region located between P1 and P2 displays promoter activity that is independent of either P1 or P2. Similar to the P2, M has promoter activity that is strongly affected by p53. M linked to P1 or P2 did not modify P1 or P2 promoter activities in either the p53-sufficient or -deficient cells. However, comparison of the activities of P1-M-P2 with P1-x-P2 (where M is deleted) suggests that in the context of the full-length *bcl-2* promoter M in a p53-dependent manner counteract the suppressive activity of P2 on P1. It is also possible that the enhanced activity of M in the absence of p53 may increase the overall activity of the full length *bcl-2* promoter and thereby counteract the suppression by P2. Because the sequences for M and P2 are highly conserved from mouse to human these promoter regions showed enhanced activity in airway cells from *p53*^{-/-} compared to those from *p53*^{+/+} mice (data not shown). However, because the P1 sequence is not conserved its activity in mouse cells is low is not affected by M or P2 in cells from *p53*^{+/+} and *p53*^{-/-} mice. We show that p53 directly interacts within the 140 bp region upstream of the open reading frame within P2. Whether p53 interacts with transcription factors complexes that bind to sequences within M or whether p53 directly interacts with M will be studied in the future.

P1, P2, and M display independent promoter activities that are not additive suggesting the existence of as yet unknown interactions between P1, M, and P2 as part of the full-length promoter. It is possible that individual promoter regions become activated based on the needs of the cell. Current studies are evaluating the activities of these promoter regions when cells are exposed to various cytokines. Our studies and those of others (Seto et al., 1988) show that P1 is the main transcriptional promoter, most analyses of *bcl-2* gene expression have focused on this promoter (Wilson et al., 1996; Pugazhenthil et al., 1999; Pugazhenthil et al., 2000). It is generally believed that P1 and P2 control transcription (Seto et al., 1988; Young and Korsmeyer, 1993). Studies performed mostly in hematopoietic cell lines and using either the P1 or P2 promoter have identified many transcription factors that affect transcription through interaction at different positive and negative regulatory elements (Lang et al., 2005). Most of these studies, however, do not involve reporter gene analysis driven by the full-length P1-M-P2 promoter. Our studies show that conclusions drawn from the individual *bcl-2* promoter regions are different from those of the full-length promoter and may not provide the complete picture of the mechanisms regulating *bcl-2* expression. Investigating the full-length *bcl-2* promoter may be necessary to unravel the complexity of *bcl-2* gene regulation.

For all constructs tested the H1299 cells showed approximately 10-fold more activity than the p53-deficient SaOS-2 cells or the p53-sufficient A549 or Calu-3 cells. It is possible that H1299 cells have increased levels of transcription factors driving the various *bcl-2* promoter regions. Because the ratio of P2 to P1 promoter activities appears to be similar in the p53-deficient SaOS-2 and H1299 cells we conclude that transcription factors other than p53 may affect transcription from P2 and M. Whether increased levels of transcription factors are responsible for increased promoter activity will be studied in the future.

In summary, our findings reveal that the P2 region interacts with p53 and its promoter activity is suppressed by p53. However, in the context of the full-length promoter, P2 suppresses P1 promoter activity in a p53-independent manner. Furthermore, we show that M, a previously unexplored region has promoter activity that is also suppressed by p53. Our findings show that the role of M in the context of the full-length promoter may be to counteract the suppressive activity of P2 on P1 and thereby affecting *bcl-2* expression in a p53-dependent manner. Thus, transcription of the *bcl-2* gene is regulated in a p53-dependent and -independent manner. These

findings may have implications for therapies that are geared towards inhibiting *bcl-2* gene expression and inducing apoptosis in tumorigenic or hyperplastic airway epithelial cells (Webb et al., 1997; Manion and Hockenbery, 2003).

5. Acknowledgements

The authors thank Kimberly Ortega for technical assistance on certain experiments. These studies were supported by grants from the National Institutes of Health (HL68111 and ES09237 to YT) and the NM NIEHS Center, P30 ES-012072.

Conflict of interest statement - None declared.

List of Abbreviations

ANOVA, analysis of variance; ChIP, chromatin immunoprecipitation; NRE, negative regulatory element; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SEM, standard error of the mean.

References

- Bredow S, Lewin M, Hofmann B, Marecos E, Weissleder R. Imaging of tumour neovasculature by targeting the TGF-beta binding receptor endoglin. *Eur J Cancer* 2000;36:675–81. [PubMed: 10738134]
- Budhram-Mahadeo V, Morris PJ, Smith MD, Midgley CA, Boxer LM, Latchman DS. p53 suppresses the activation of the *Bcl-2* promoter by the Brn-3a POU family transcription factor. *J Biol Chem* 1999;274:15237–44. [PubMed: 10329733]
- Catz SD, Johnson JL. Transcriptional regulation of *bcl-2* by nuclear factor kappa B and its significance in prostate cancer. *Oncogene* 2001;20:7342–51. [PubMed: 11704864]
- Chen HM, Boxer LM. Pi 1 binding sites are negative regulators of *bcl-2* expression in pre-B cells. *Mol Cell Biol* 1995;15:3840–7. [PubMed: 7791791]
- el-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW, Vogelstein B. Definition of a consensus binding site for p53. *Nat Genet* 1992;1:45–9. [PubMed: 1301998]
- Harn HJ, Ho LI, Liu CA, Liu GC, Lin FG, Lin JJ, Chang JY, Lee WH. Down regulation of *bcl-2* by p53 in nasopharyngeal carcinoma and lack of detection of its specific t(14;18) chromosomal translocation in fixed tissues. *Histopathology* 1996;28:317–23. [PubMed: 8732340]
- Harris JF, Fischer MJ, Hotchkiss JA, Monia BP, Randell SH, Harkema JR, Tesfaigzi Y. Bcl-2 sustains increased mucous and epithelial cell numbers in metaplastic airway epithelium. *Am J Respir Crit Care Med* 2005;171:764–772. [PubMed: 15618464]
- Hockenbery D, Nunez G, Millman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 1990;348:334–6. [PubMed: 2250705]
- Hoehner JC, Gestblom C, Olsen L, Pahlman S. Spatial association of apoptosis-related gene expression and cellular death in clinical neuroblastoma. *Br J Cancer* 1997;75:1185–94. [PubMed: 9099968]
- Imbriano C, Gurtner A, Cocchiarella F, Di Agostino S, Basile V, Gostissa M, Dobbelsstein M, Del Sal G, Piaggio G, Mantovani R. Direct p53 transcriptional repression: in vivo analysis of CCAAT-containing G2/M promoters. *Mol Cell Biol* 2005;25:3737–51. [PubMed: 15831478]
- Lang G, Gombert WM, Gould HJ. A transcriptional regulatory element in the coding sequence of the human *bcl-2* gene. *Immunology* 2005;114:25–36. [PubMed: 15606792]
- Lee WY, Jin YT, Tzeng CC. Reciprocal expression of Bcl-2 and p53 in breast ductal carcinoma. *Anticancer Res* 1996;16:3007–12. [PubMed: 8917421]
- Manion MK, Hockenbery DM. Targeting BCL-2-related proteins in cancer therapy. *Cancer Biol Ther* 2003;2:S105–14. [PubMed: 14508087]
- Miyashita T, Harigai M, Hanada M, Reed JC. Identification of a p53-dependent negative response element in the *bcl-2* gene. *Cancer Res* 1994a;54:3131–5. [PubMed: 8205530]
- Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B, Reed JC. Tumor suppressor p53 is a regulator of *bcl-2* and bax gene expression in vitro and in vivo. *Oncogene* 1994b;9:1799–805. [PubMed: 8183579]

- Pugazhenth S, Miller E, Sable C, Young P, Heidenreich KA, Boxer LM, Reusch JE. Insulin-like growth factor-I induces *bcl-2* promoter through the transcription factor cAMP-response element-binding protein. *J Biol Chem* 1999;274:27529–35. [PubMed: 10488088]
- Pugazhenth S, Nesterova A, Sable C, Heidenreich KA, Boxer LM, Heasley LE, Reusch JE. Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. *J Biol Chem* 2000;275:10761–6. [PubMed: 10753867]
- Reed JC. Bcl-2 family proteins. *Oncogene* 1998;17:3225–36. [PubMed: 9916985]
- Reed JC. Dysregulation of apoptosis in cancer. *J Clin Oncol* 1999;17:2941–53. [PubMed: 10561374]
- Seto M, Jaeger U, Hockett RD, Graninger W, Bennett S, Goldman P, Korsmeyer SJ. Alternative promoters and exons, somatic mutation and deregulation of the Bcl-2-Ig fusion gene in lymphoma. *Embo J* 1988;7:123–31. [PubMed: 2834197]
- Tesfaigzi Y. Roles of Apoptosis in Airway Epithelia. *Am J Respir Cell Mol Biol* 2006;34:537–547. [PubMed: 16439804]
- Tesfaigzi Y, Fischer MJ, Martin AJ, Seagrave J. Bcl-2 in LPS- and allergen-induced hyperplastic mucous cells in airway epithelia of Brown Norway rats. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L1210–L1217. [PubMed: 11076811]
- Vaux DL, Cory S, Adams JM. *Bcl-2* gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* 1988;335:440–2. [PubMed: 3262202]
- Webb A, Cunningham D, Cotter F, Clarke PA, di Stefano F, Ross P, Corbo M, Dziewanowska Z. BCL-2 antisense therapy in patients with non-Hodgkin lymphoma. *Lancet* 1997;349:1137–41. [PubMed: 9113013]
- Wilson BE, Mochon E, Boxer LM. Induction of *bcl-2* expression by phosphorylated CREB proteins during B-cell activation and rescue from apoptosis. *Mol Cell Biol* 1996;16:5546–56. [PubMed: 8816467]
- Wu Y, Mehew JW, Heckman CA, Arcinas M, Boxer LM. Negative regulation of *bcl-2* expression by p53 in hematopoietic cells. *Oncogene* 2001;20:240–51. [PubMed: 11313951]
- You Y, Richer EJ, Huang T, Brody SL. Growth and differentiation of mouse tracheal epithelial cells: selection of a proliferative population. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L1315–21. [PubMed: 12388377]
- Young RL, Korsmeyer SJ. A negative regulatory element in the *bcl-2* 5'-untranslated region inhibits expression from an upstream promoter. *Mol Cell Biol* 1993;13:3686–97. [PubMed: 8388542]

Figure 1.

Bcl-2 promoter constructs. Within the full-length-promoter (P1-M-P2) positions for promoters P1-M, P1-x-P2, P1, M, and M-P2 as well as region P2 are shown; arrows indicate the transcriptional start sites of P1 and P2. All *bcl-2* promoter fragments are upstream of the reporter gene firefly luciferase (Luc). In construct P1-M-ΔP2, deletion of 140 bp within P2 yielded a construct without CCAAT- and TATA-boxes. The positions of the PCR primers, recognizing the P2 promoter (s) and luciferase gene (as), are shown; amplification of full-length P2-containing constructs yields a 205 bp-fragment, while amplification of deletion construct P1-M-ΔP2 yields a 65 bp-fragment.

A

B

Figure 2.

(A) *Bcl-2* promoter activity in p53-sufficient and -deficient cells. P1-M-P2, P1, and P2 were transfected into p53-sufficient (A549, Calu-3) and p53-deficient (SaOS-2, H1299) cell lines using FuGENE 6. Cells were lysed 48h later and promoter activity analyzed. (B) Mouse airway epithelial cells, isolated from tracheas of three *p53*^{+/+} (*p53*^{+/+}) and *p53*^{-/-} (*p53*^{-/-}) mice respectively were cultured on Transwell membranes were transfected with either P1 or P2 promoter constructs as described in Materials and Methods. Firefly luciferase (FF) activity was measured and normalized to that of co-transfected Renilla (RL) luciferase, controlled by the human thymidine kinase promoter. Values represent the means $\pm$ SEM ($n = 3$ independent transfections performed in duplicate for each construct) (* $p < 0.05$).

A

B

Figure 3.

(A) Interaction of p53 with P2 *in vivo*. A549 cells were transfected with P1, P2 and P1-M-ΔP2. Purified cross-linked chromatin was treated with either no antibody (-) or 2 μg anti-p53 specific antibody (+). After immunoprecipitation, DNA was extracted and PCR (28 cycles) performed with the primer set described in Materials and Methods/Figure 1. Amplified products were resolved in 2% agarose gels and stained with ethidium bromide. NT: genomic DNA from non-transfected cells; i: input DNA from transfected cells; B: buffer control; W: water control; M: DNA molecular weight markers (1 kB Plus Ladder, Invitrogen; Carlsbad, CA). (B) Competition of P2 promoter activity in p53-sufficient (A549) and -deficient (H1299) cells. Activities for P1-M-P2, P1, and P2 were determined in A549 and H1299 cells co-transfected with either P2-TA or the empty TA vector as control. Values for the respective promoter constructs in P2-TA transfected cells were normalized to those transfected with empty-TA. Values represent means ± SEM (n = 3 independent transfections performed in duplicate (*p<0.05 vs empty-TA)).

A

B

C

Figure 4.

Analysis of M promoter activity in p53-sufficient and -deficient cells (A) Comparison of P1, M, and P1-M promoter activities. (B) Comparison of the promoter activities of P1, P2, and M-P2. (C) Comparison of the promoter activities of P1, P1-M-P2, and P1-x-P2 that lacks the M region. Promoter constructs were transfected FF luciferase activity normalized to that of RL luciferase as described in Materials and Methods. Values represent means $\pm$ SEM (n = 3 independent transfections performed in duplicate) (*p<0.05 vs P1).