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Modification of Alternative Splicing of Bcl-x Pre-mRNA in Prostate and Breast Cancer Cells

ANALYSIS OF APOPTOSIS AND CELL DEATH*

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There is ample evidence that deregulation of apoptosis results in the development, progression, and/or maintenance of cancer. Since many apoptotic regulatory genes (*e.g. bcl-x*) code for alternatively spliced protein variants with opposing functions, the manipulation of alternative splicing presents a unique way of regulating the apoptotic response. Here we have targeted oligonucleotides antisense to the 5′-splice site of *bcl-x$_L$*, an anti-apoptotic gene that is overexpressed in various cancers, and shifted the splicing pattern of Bcl-x pre-mRNA from Bcl-x$_L$ to Bcl-x$_S$, a pro-apoptotic splice variant. This approach induced significant apoptosis in PC-3 prostate cancer cells. In contrast, the same oligonucleotide treatment elicited a much weaker apoptotic response in MCF-7 breast cancer cells. Moreover, although the shift in Bcl-x pre-mRNA splicing inhibited colony formation in both cell lines, this effect was much less pronounced in MCF-7 cells. These differences in responses to oligonucleotide treatment were analyzed in the context of expression of Bcl-x$_L$, Bcl-x$_S$, and Bcl-2 proteins. The results indicate that despite the presence of Bcl-x pre-mRNA in a number of cell types, the effects of modification of its splicing by antisense oligonucleotides vary depending on the expression profile of the treated cells.

Apoptosis, or programmed cell death, is a highly regulated process controlled by numerous genes that determine a proper response to death signals (1–4); the relative levels of expression of pro- and anti-apoptotic genes appear to be particularly important (5–9). Deregulation of apoptosis, which contributes to the development, progression, and/or maintenance of cancer (3, 4, 10), is frequently caused by mechanisms that alter splicing of regulatory genes (11, 12). Therefore, this work focused on the modification of alternative splicing of *bcl-x*, a member of the *bcl-2* family of apoptotic genes that play crucial roles in both inhibiting and activating the apoptotic response to cellular insults (1, 2). Bcl-x is alternatively spliced to produce two distinct mRNAs and two variant proteins, Bcl-x$_L$ and Bcl-x$_S$, that have antagonistic functions; the longer, 241-amino acid protein (Bcl-x$_L$) inhibits apoptosis, whereas the shorter, 178-amino acid protein (Bcl-x$_S$) activates it (13).

Bcl-x$_L$ is highly expressed in many types of cancers, including multiple myeloma (14), small cell lung carcinoma (15), and breast cancer (16). High Bcl-x$_L$ expression levels have been associated with an increased risk of metastasis in breast cancer (16) as well as with an increased resistance to apoptosis induced by methotrexate and 5-fluorouracil (17) and etoposide and cisplatin (18). Additionally, an immunohistochemical study (19) showed that Bcl-x, presumably Bcl-x$_L$, was expressed in 100% of prostate tumors and that its immunointensity was stronger in higher grade metastases.

Bcl-x$_S$ antagonizes the pro-survival properties of Bcl-x$_L$ (13, 20) and appears to induce apoptosis. For example, high levels of Bcl-x$_S$ induced apoptosis in cancer cells from patients with colon and stomach cancers (21) as well as in human mammary tumors in nude mice (22). However, overexpression of Bcl-x$_S$ did not cause apoptosis in an established breast cancer cell line; the latter cells became apoptotic only after additional treatment with the chemotherapeutic agents Taxol and etoposide (23).

Work from this laboratory showed that antisense oligonucleotides could be used to modify the splicing patterns of various genes in cell culture (24–27). We therefore hypothesized that blocking the alternative 5′-splice site in intron 2 of Bcl-x with an antisense oligonucleotide should shift splicing from Bcl-x$_L$ to Bcl-x$_S$ mRNA, thereby increasing the level of pro-apoptotic Bcl-x$_S$ protein and decreasing the level of its anti-apoptotic isoform, Bcl-x$_L$. We show here that this shift in splicing could indeed be accomplished and that it induced apoptotic markers in the prostate cancer cell line PC-3. However, in the breast cancer cell line MCF-7, shifting splicing from Bcl-x$_L$ to Bcl-x$_S$ and induction of apoptosis were much less efficient. Differences in the responses to oligonucleotide treatment were also evident in the inhibition of cell growth of the two cell lines.

EXPERIMENTAL PROCEDURES

Cells—PC-3 cells were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 supplemented with 10% fetal calf serum. MCF-7 cells were cultured in modified essential medium supplemented with 10% fetal calf serum, 1× sodium pyruvate (Life Technologies, Inc.), 1× nonessential amino acids (Sigma), and 10 μg/ml insulin. Twenty-four hours prior to oligonucleotide treatment, the cells were plated in 2 ml of medium in 6-well plates at a density of 2×10^5 cells/well. For experiments requiring estrogen-free medium, MCF-7 cells were cultured in phenol red-free medium containing 10% (4 days) and then 3% (3 days) charcoal-stripped fetal calf serum (Hyclone Laboratories, Logan, UT) (28, 29).

Oligonucleotide Treatment—2′-*O*-Methyl-modified oligoribonucleoside phosphorothioate 18-mers antisense to the 5′-splice site of Bcl-x$_L$ (5′-Bcl-x AS, ACCCAGCCGCCGUUCUCC) and to the 3′-splice site of exon III in Bcl-x pre-mRNA (3′-Bcl-x AS, GUUCCACAAAAGUAUCCU) were used. Oligonucleotides with randomized and anti-β-globin se-

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quences (24) were used as negative controls. All oligonucleotides were synthesized and purified by Hybridon, Inc. (Milford, MA) and Trilink Biotechnologies, Inc. (San Diego, CA). The cells were treated with oligonucleotide·DMRIE-C Reagent (8 μg/ml; Life Technologies, Inc.) cationic lipid complexes according to the manufacturer's directions at the concentrations indicated on the figures. Ten hours post-treatment, the medium was replaced with fresh medium, and the cells were cultured for the indicated times. Thapsigargin (0.5 μM final concentration; Sigma) was added directly to the medium of MCF-7 cells 24 h post-transfection.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)[1]—Oligonucleotide-treated cells were lysed in 1 ml of TRI-reagent (Molecular Research Center, Inc., Cincinnati, OH), and total RNA was isolated. RNA (200 ng) was used in RT-PCR with rTth enzyme (PerkinElmer Life Sciences) in the presence of 0.2 μCi of [α-^{32}P]dATP. Both procedures followed the manufacturers' protocols. The reverse transcription reaction was carried out at 70 °C for 15 min, followed by PCR: one cycle of 95 °C for 3 min; 22 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 7 min. The forward and reverse primers used were GCATTGTTCCCATAGAGTTCC and GCATTGTTCCCATAGAGTTCC, respectively. Under these conditions, the linear concentration-dependent response of PCR was maintained (data not shown). The resulting products (Bcl-x_L, 300 bp; and Bcl-x_S, 162 bp) were separated on 8% nondenaturing polyacrylamide gels, and the gels were autoradiographed with Kodak BioMax film. All autoradiograms were captured by a Dage-MTI CCD72 video camera, and the images were processed using NIH IMAGE Version 1.61 software. NIH IMAGE was also used to quantify the density of the bands. The percentage of Bcl-x_S in each lane was determined by dividing the intensity of the 162-bp band (Bcl-x_S) by the total intensities of the 300-bp (Bcl-x_L) and 162-bp (Bcl-x_S) bands. The calculations reflect the fact that the number of radioactive adenosine nucleotides in the Bcl-x_L band is 1.2 times higher than that in the Bcl-x_S band. Thus, the percent of correction is higher than appears from the autoradiograms.

Protein Analysis—Transfected cells were harvested at the indicated time points in radioimmune precipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) and a mixture of protease inhibitors (Sigma). Total protein (100 μg for Bcl-x_S, 10 μg for Bcl-x_L and Bcl-2, and 75 μg for PARP) from the cells was electrophoresed on a 15% (for Bcl-2, Bcl-x_L, and Bcl-x_S) or 8% (for PARP) SDS-polyacrylamide gel and electrotransferred to polyvinylidene difluoride membranes. Equal gel loading and transfer of protein were confirmed by staining the membranes with Ponceau S. Membranes were blocked for 1 h with BLOTTO (5% nonfat powdered milk in Tris-buffered saline/Tween) and incubated for 1 h at room temperature with rabbit anti-Bcl-x_L polyclonal antibody (1:1000 dilution; Transduction Laboratories, Lexington, KY), mouse anti-Bcl-2 monoclonal antibody (1:250 dilution; Transduction Laboratories), or rabbit anti-PARP polyclonal antibody (1:1000 dilution; Cell Signaling Technology, Beverly, MA), followed by 1 h of incubation with horseradish peroxidase-conjugated anti-rabbit (1:5000 dilution; Bio-Rad) or anti-mouse (1:1000 dilution; Amersham Pharmacia Biotech) secondary antibodies. Blots were developed with ECL Plus reagents (Amersham Pharmacia Biotech) and exposed to Kodak film. Bcl-x_L, Bcl-x_S, Bcl-2, and cleaved PARP proteins migrated at ~30, 21, 26, and 89 kDa, respectively. The densities of the resulting bands were quantified using NIH IMAGE Version 1.61 software.

Fluorescence-activated Cell Sorting (FACS) Analysis—At the indicated times after oligonucleotide treatment, cells were resuspended by trypsinization, and aliquots were removed for RT-PCR analysis (see above) and for colony formation assays (see below). The remaining cells were washed twice with cold 1× phosphate-buffered saline, fixed in cold 70% ethanol, and stored at −20 °C for at least 24 h. They were then washed with 1× phosphate-buffered saline and treated with 20 μM propidium iodide and 1 mg/ml RNase A in 1× phosphate-buffered saline for 30 min in the dark. Cells were analyzed by flow cytometry using a Becton Dickinson FACSort for two measures of apoptosis: subdiploid DNA and loss of cell volume (30–32).

Colony Formation Assay—Aliquots of trypsinized cell suspensions were seeded on 100-mm plates at 5×10^2 for PC-3 cells and 1×10^3 for MCF-7 cells. After 10 days under normal culture conditions, cells were stained with 5% methylene blue (Sigma) in 50% ethanol for 10 min. Colonies of >50 cells were counted.

[1] The abbreviations used are: RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s); PARP, poly(ADP-ribose) polymerase; FACS, fluorescence-activated cell sorting.

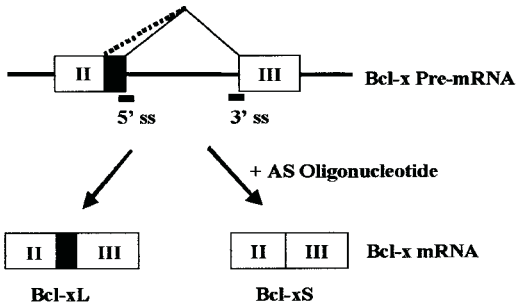

FIG. 1. **Alternative splicing of Bcl-x pre-mRNA.** Use of the downstream or upstream alternative 5′-splice site within exon II yields the anti-apoptotic Bcl-x_L or pro-apoptotic Bcl-x_S isoform, respectively. The *thin line* indicates the Bcl-x_L splicing pathway, predominant in many cancer cells. The *dotted line* indicates the splicing pathway for Bcl-x_S mRNA. *Boxes*, exons; *thick lines*, introns; *solid black boxes*, the 189-nucleotide portion of exon II included in Bcl-x_L; *thick bars* below the pre-mRNA, antisense oligonucleotides. The oligonucleotide antisense to the 5′-splice site (5′-Bcl-x AS), but not that antisense to the 3′-splice site (3′-Bcl-x AS), of Bcl-x pre-mRNA induced a shift in splicing from Bcl-x_L to Bcl-x_S.

Statistical Analysis—Numerical data were analyzed by one-way analysis of variance and *post hoc* Student-Neuman-Keuls tests using the statistical software program STATVIEW. The significance level for all analyses was 5%.

RESULTS

Shift in Splicing from Bcl-x_L to Bcl-x_S in Oligonucleotide-treated PC-3 Cells—PC-3 cells were treated with increasing amounts of 5′-Bcl-x AS, a 2′-*O*-methyl-modified oligoribonucleoside phosphorothioate 18-mer targeted to the downstream alternative 5′-splice site in Bcl-x pre-mRNA. Splicing at this site led to Bcl-x_L mRNA and protein; alternative splicing at the upstream site resulted in the Bcl-x_S splice variant (Fig. 1). The 2′-*O*-methyl-modified oligonucleotide was chosen because it is resistant to nucleases, does not induce degradation of RNA in the RNA-oligonucleotide hybrid by RNase H (33), and binds to the target sequence with high T_m (34). These properties predict that the oligonucleotide should block the targeted splice site and induce a shift in the splicing pathways from Bcl-x_L to Bcl-x_S mRNA.

RT-PCR analysis (see "Experimental Procedures") of total cell RNA 24 h post-treatment showed that 5′-Bcl-x AS treatment led to a dose-dependent shift in splicing from the Bcl-x_L to Bcl-x_S pathway as indicated by a shift in the ratios of the respective mRNAs (Fig. 2*A*, *upper panel*, *lanes 2–7*). At the highest concentrations of the 5′-Bcl-x AS oligonucleotide, the level of Bcl-x_S reached ~65% of the total amount of Bcl-x_S and Bcl-x_L isoforms (Fig. 2*A*, *lower panel*, *lane 7*). There was no shift in Bcl-x pre-mRNA splicing in cells treated with an oligonucleotide with a randomized sequence (Fig. 2*A*, *lanes 8–11*). After a single treatment of PC-3 cells with 0.08 μM 5′-Bcl-x AS oligonucleotide, a maximal shift in splicing occurred at 12 h post-treatment and persisted, with a slight decrease, for at least 72 h (Fig. 2*B*, *lanes 3–6*). This decrease was presumably due to dilution of the oligonucleotide and/or instability of the oligonucleotide and Bcl-x_S mRNA in dividing cells. As expected, despite prolonged culture of the cells, the randomized oligonucleotide had no effect on the Bcl-x_L/Bcl-x_S mRNA ratio (Fig. 2*B*, *lanes 7–11*).

Since a shift in the splicing pattern of Bcl-x pre-mRNA from Bcl-x_L to Bcl-x_S should change the Bcl-x_L/Bcl-x_S protein ratio, we analyzed total protein from the PC-3 cell line by immunoblotting and probing with an antibody expected to detect both splice variants of the Bcl-x protein. This analysis showed a time-dependent decrease in Bcl-x_L protein (Fig. 3, *upper panel*, *lanes 2–5*) and an increase in Bcl-x_S protein (*lower panel*, *lanes*

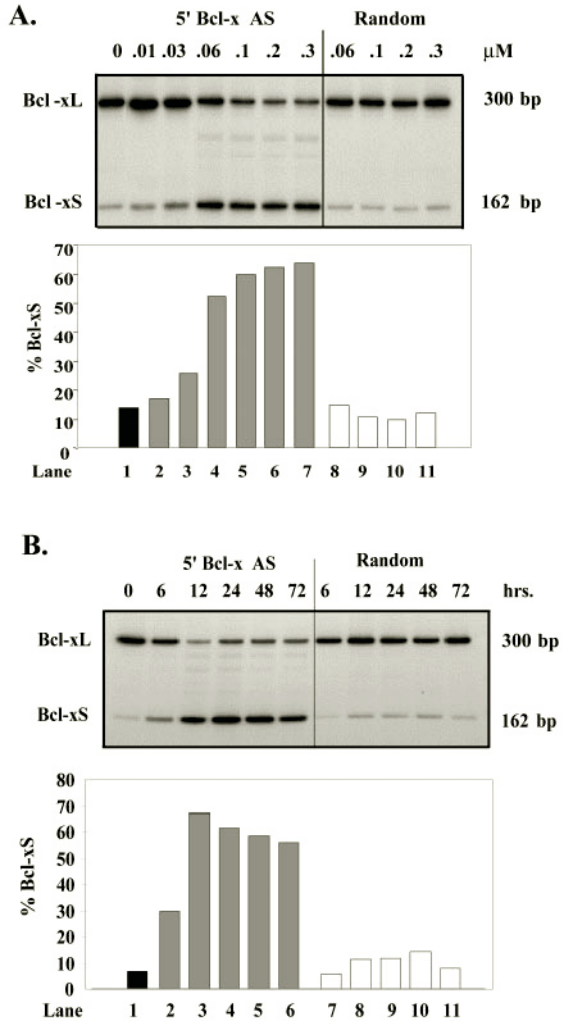

FIG. 2. **Shift in splicing from Bcl-x_L to Bcl-x_S by treatment of PC-3 cells with 5′-Bcl-x AS.** The results from the analysis of total cellular RNA by RT-PCR are shown. *A*: *upper panel*, dose dependence of cells treated for 24 h. *Lane 1*, mock-transfected cells; *lanes 2–7*, cells transfected with 5′-Bcl-x AS; *lanes 8–11*, cells transfected with an oligonucleotide with randomized sequence. The lengths of the PCR products (in base pairs) are indicated to the right. *Lower panel*, quantitative analysis of the results. *Black bar*, mock-transfected cells; *gray bars*, 5′-Bcl-x AS oligonucleotide-transfected cells; *white bars*, randomized oligonucleotide-treated cells. *B*: *upper panel*, time course. *Lane 1*, mock-transfected cells; *lanes 2–6*, RNA isolated from 5′-Bcl-x AS oligonucleotide (0.08 μM)-transfected cells 6, 12, 24, 48, and 72 h post-transfection, respectively; *lanes 7–11*, cells transfected with the randomized oligonucleotide (0.08 μM). *Lower panel*, quantitation of the results. Designations are as the same as described for *A*.

2–5) in cells treated with 0.08 μM 5′-Bcl-x AS oligonucleotide, but not in cells treated with the randomized oligonucleotide (*lower panel*, *lanes 6–9*).

Apoptosis and Death of PC-3 Cells Caused by a Shift in Splicing from Bcl-x_L to Bcl-x_S—To determine whether increasing the levels of Bcl-x_S protein increased apoptosis of 5′-Bcl-x AS oligonucleotide-treated PC-3 cells, the cells were analyzed by FACS for two measures of apoptosis: subdiploid DNA and loss of cell volume. As illustrated in Fig. 4*A*, the population of cells that had degraded their DNA (shown in *green* in the *left panels* and to the left of the *bars* in the *right panels*) also exhibited a decreased cell size (*lower left corners* in the *left panels*). Quantitation of the results of multiple experiments showed that treatment with 0.01, 0.03, and 0.08 μM 5′-Bcl-x AS oligonucleotide resulted in 6.4, 11.2, and 19.8% of the PC-3 cells, respectively, exhibiting subdiploid DNA (Fig. 4*B*); the

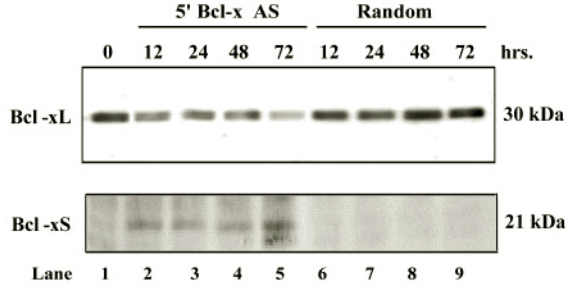

FIG. 3. **Western blot analysis of Bcl-x proteins from PC-3 cells transfected with 0.08 μM 5′-Bcl-x AS.** The times post-transfection are indicated above the lanes. *Upper panel*, Bcl-x_L protein; *lower panel*, Bcl-x_S protein. *Lanes 2–5*, cells treated with 5′-Bcl-x AS; *lanes 6–9*, cells treated with the randomized oligonucleotide. See "Experimental Procedures" for details.

latter result was statistically significant ($p < 0.0001$, analysis of variance and Student-Neuman-Keuls tests) in comparison with mock-treated cells (4.4%) or with cells transfected with 0.8 μM randomized oligonucleotide (5.0%). Similarly, treatment with the 5′-Bcl-x AS oligonucleotide resulted in a dose-dependent loss of cell volume; at 0.08 μM 5′-Bcl-x AS, the effect (20.3%) was statistically significant ($p < 0.0001$) relative to either mock-treated (5.2%) or randomized oligonucleotide-treated (6.0%) cells (data not shown). Thus, by two different criteria and consistent with the RT-PCR and immunoblot results, the shift in splicing of Bcl-x pre-mRNA from Bcl-x_L to Bcl-x_S led to a dose-dependent increase in apoptosis of PC-3 cells.

The ultimate goal of shifting splicing from the Bcl-x_L to Bcl-x_S isoform is to induce cell death. Since it has been argued that apoptotic markers, especially in cells with mutated p53, underestimate the killing potential of cytotoxic drugs (35), we have analyzed the effects of 5′-Bcl-x AS treatment of PC-3 cells on cell survival. The colony formation assay showed statistically significant cell death at 0.01, 0.03, and 0.08 μM 5′-Bcl-x AS oligonucleotide compared with mock- or randomized oligonucleotide-transfected cells (Fig. 4*C*). At these oligonucleotide concentrations, the number of surviving cells was reduced 2.0-, 2.6-, and 4.4-fold, respectively, relative to mock treatment and 1.7-, 1.8-, and 3.8-fold relative to randomized oligonucleotide treatment. Thus, the long-term effects of the treatment were even more pronounced than those detected by the short-term assays of apoptotic markers.

Inefficient Induction of Apoptosis by Down-regulation of Bcl-x_L Pre-mRNA in PC-3 Cells—Shifting splicing of Bcl-x pre-mRNA should presumably lead to a more efficient induction of apoptosis than down-regulation of Bcl-x_L because the former approach leads to simultaneous down-regulation of anti-apoptotic Bcl-x_L and up-regulation of pro-apoptotic Bcl-x_S. To test this hypothesis, PC-3 cells were treated with an oligonucleotide targeted to the 3′-splice site of exon III in Bcl-x pre-mRNA (3′-Bcl-x AS; see Fig. 1). In contrast to 5′-Bcl-x AS, this oligonucleotide should simultaneously decrease the level of Bcl-x_L and Bcl-x_S proteins because the 3′-splice site is common to both Bcl-x_L and Bcl-x_S splice variants.

As predicted, at 0.08 μM, the 3′-Bcl-x AS oligonucleotide led to a decrease in the levels of Bcl-x_L protein (Fig. 5*A*, *upper panel*, *lane 2*). The levels of Bcl-x_S protein appeared unaffected (Fig. 5*A*, *lower panel*, *lane 2*) and equal to that in mock-treated or control cells (*lanes 1* and *4*, respectively). In agreement with the results shown in Fig. 3, 5′-Bcl-x AS decreased Bcl-x_L levels (Fig. 5*A*, *upper panel*, *lane 3*), but increased Bcl-x_S protein levels (*lower panel*, *lane 3*). FACS analysis of the oligonucleotide-treated cells showed that 3′-Bcl-x AS was approximately half as efficient as 5′-Bcl-x AS in inducing apoptosis (Fig. 5*B*). These results confirm that apoptosis of PC-3 cells is more

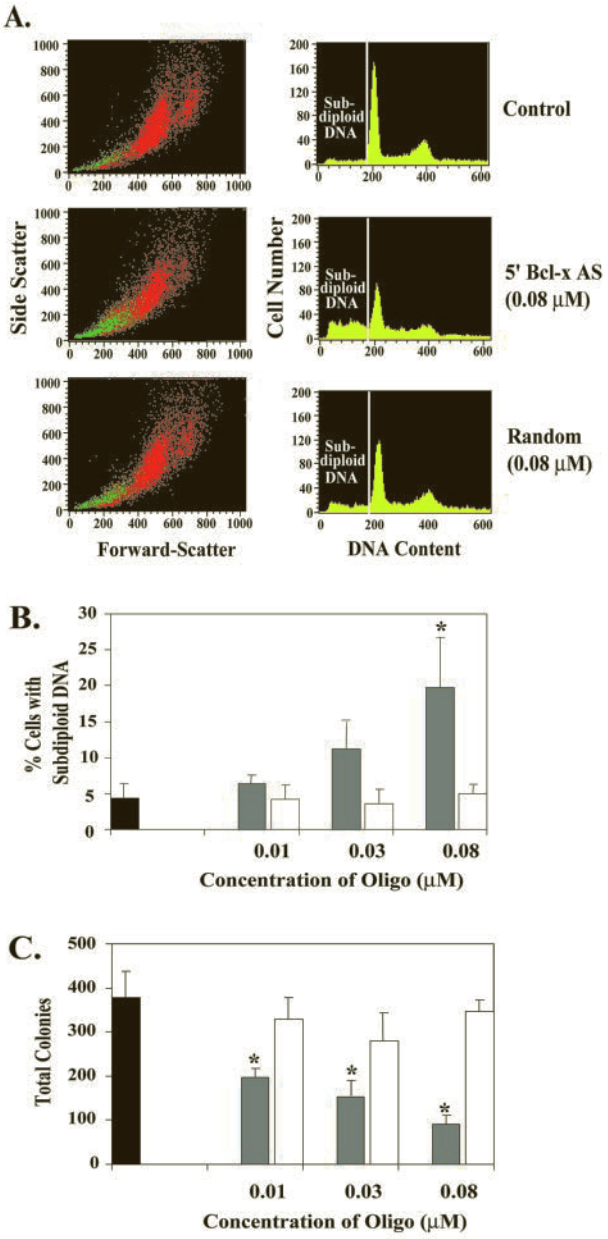

FIG. 4. **Apoptosis of PC-3 cells treated with 5′-Bcl-x AS (0.08 μM) for 36 h.** *A*, FACS analysis plots. The *left panels* show dot plots of forward scatter (cell size) *versus* side scatter (cell granularity). Cells that have degraded their DNA are shown in *green* and are smaller in size. The *right panels* show the DNA histograms (with subdiploid DNA to the left of the *bars*). *B*, quantitation of subdiploid DNA. The *asterisk* indicates a statistically significant difference from mock and randomized oligonucleotide treatments ($p < 0.0001$; significance level = 5%). Results are from at least three independent experiments. *Black bar*, mock-transfected cells; *gray bars*, 5′-Bcl-x AS oligonucleotide-transfected cells; *white bars*, random oligonucleotide-transfected cells. *C*, inhibition of colony formation in cells treated with the 5′-Bcl-x AS oligonucleotide ($p < 0.0001$; significance level = 5%). The number of experiments and designations are the same as described for *B*.

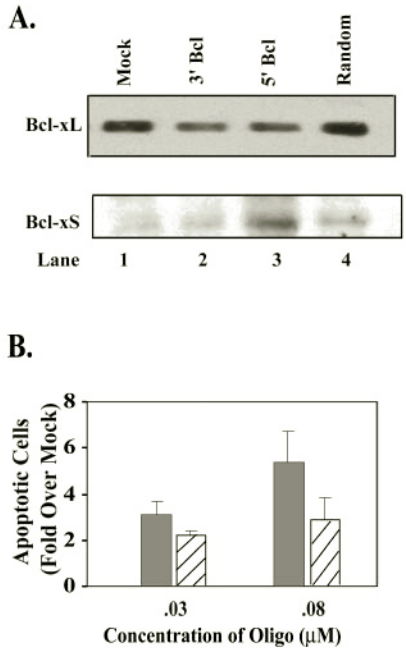

FIG. 5. **Inefficient induction of apoptosis in PC-3 cells by 3′-Bcl-x AS targeted to the 3′-splice site of Bcl-x pre-mRNA.** *A*, Western blot analysis of Bcl-x proteins from PC-3 cells transfected for 36 h with 0.08 μM 3′- and 5′-Bcl-x AS oligonucleotides. *Upper panel*, Bcl-x_L protein; *lower panel*, Bcl-x_S protein. *Lane 1*, mock-transfected cells; *lane 2*, 3′-Bcl-x AS oligonucleotide-treated cells; *lane 3*, 5′-Bcl-x AS oligonucleotide-treated cells; *lane 4*, randomized oligonucleotide (0.08 μM)-treated cells. *B*, FACS analysis of subdiploid DNA in cells treated for 36 h with 3′- and 5′-Bcl-x AS oligonucleotides. *Gray bars*, treatment with 5′-Bcl-x AS; *hatched bars*, treatment with 3′-Bcl-x AS.

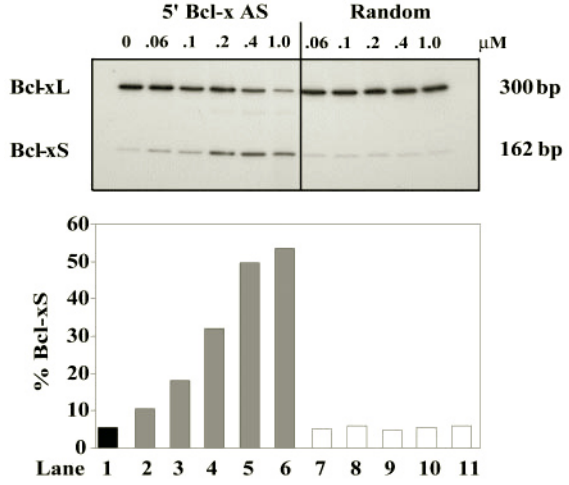

FIG. 6. **Shift in splicing from Bcl-x_L to Bcl-x_S in MCF-7 cells treated with 5′-Bcl-x AS for 24 h.** *Upper panel*, analysis of total RNA by RT-PCR. *Lane 1*, mock-transfected cells; *lanes 2–6*, 5′-Bcl-x AS oligonucleotide-treated cells; *lanes 7–11*, randomized oligonucleotide-treated cells. *Lower panel*, quantitation of the results. Designations are the same as described in the legend to Fig. 2*A*.

efficiently induced by an increase in Bcl-x_S than by a decrease in Bcl-x_L, validating the approach of modification of splicing of Bcl-x pre-mRNA.

Shift in Splicing from Bcl-x_L to Bcl-x_S in MCF-7 Cells Leads to Cell Death with Minimal Induction of Apoptosis—Since Bcl-x_L is overexpressed in ~40–60% of breast cancers (16), we also tested the 5′-Bcl-x AS oligonucleotide against a breast cancer cell line, MCF-7. Similar to PC-3 cells, treatment of MCF-7 cells with the oligonucleotide·DMRIE-C Reagent complex resulted in a dose-dependent (Fig. 6, *lanes 2–6*) and sequence-dependent (*lanes 7–11*) shift in Bcl-x pre-mRNA splicing from the Bcl-x_L to Bcl-x_S pathway; however, the effects were much less pronounced. At 0.1 μM oligonucleotide, only 18% of Bcl-x_S was generated (compared with 62% in PC-3 cells), and the maximal Bcl-x_S level (54%) was reached at 1.0 μM oligonucleotide, *i.e.* at a concentration almost 10 times higher than that needed for PC-3 cells. As a consequence of the shift in splicing, the level of Bcl-x_L protein decreased, and that of Bcl-x_S protein increased (see Fig. 10, *A* and *B*, respectively, *lane 5*). The effects of the 5′-Bcl-x AS oligonucleotide persisted for up to 72 h (data not shown).

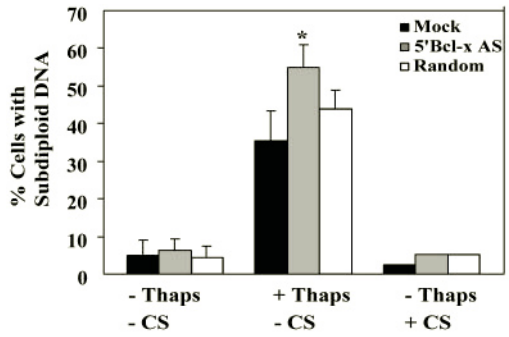

FIG. 7. **Apoptosis of MCF-7 cells treated with 5′-Bcl-x AS.** Shown are the results from FACS analysis for subdiploid DNA of MCF-7 cells transfected for 24 h with 0.4 μM 5′-Bcl-x AS, followed by a 48-h treatment with 0.5 μM thapsigargin (*Thaps*). *Left three bars*, no thapsigargin (72 h post-transfection); *middle three bars*, plus thapsigargin (48 h after thapsigargin treatment, 72 h post-transfection); *right three bars*, cells cultured in the presence of charcoal-stripped serum (*CS*; 72 h post-transfection). See "Experimental Procedures" for details. The asterisk indicates a significant difference from mock transfections ($p = 0.04$; significance level = 5%). *Black bars*, mock-transfected cells; *gray bars*, 5′-Bcl-x AS oligonucleotide-transfected cells; *white bars*, randomized oligonucleotide-transfected cells.

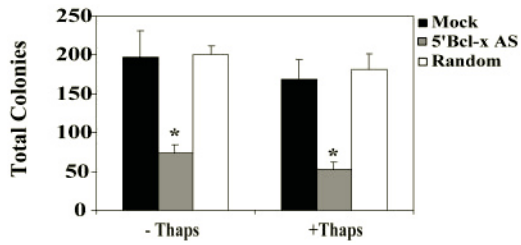

FIG. 8. **Inhibition of colony formation in MCF-7 cells treated with 5′-Bcl-x AS (0.4 μM) as well as thapsigargin (5 μM for 48 h).** *Asterisks* indicate significant difference from mock- and randomized oligonucleotide-treated cells ($p = 0.0002$; significance level = 5%). Designations are the same as described in the legend to Fig. 7. Results are from at least three independent experiments. *Thaps*, thapsigargin.

In contrast to PC-3 cells, 5′-Bcl-x AS oligonucleotide-treated MCF-7 cells did not appear to undergo significant apoptosis. Approximately 3.6, 5.2, and 5.2% of the cells exhibited subdiploid DNA (Fig. 7, *left three bars*) and loss of cell volume (data not shown) when mock-transfected or transfected with the randomized oligonucleotide or with the 5′-Bcl-x AS oligonucleotide, respectively. Interestingly, despite the lack of apoptotic markers, the colony formation assay showed that the cells transfected with the 5′-Bcl-x AS oligonucleotide formed ~2.7-fold fewer colonies than the negative control cells (Fig. 8, *left three bars*).

Since the MCF-7 cells treated with 5′-Bcl-x AS alone did not exhibit subdiploid DNA or loss of cell volume, we sought to determine if the increased Bcl-x_L/Bcl-x_S isoform ratio generated by the oligonucleotide treatment of the cells sensitized them to apoptotic inducers such as thapsigargin. Thapsigargin is known to induce apoptosis by depleting the intracellular calcium stores without increasing the influx of extracellular calcium (36). This mechanism was shown to be inhibited by anti-apoptotic proteins such as Bcl-2 (37) and Bcl-x_L (38) and should be restored if the ratio of Bcl-x_S to Bcl-x_L and Bcl-2 were significantly increased.

Twenty-four hours after transfection with the oligonucleotide, the cells were treated with 0.5 μM thapsigargin for 48 h. Relative to mock-transfected cells, 5′-Bcl-x AS combined with thapsigargin treatment resulted in a statistically significant increase in the percentage of cells with subdiploid DNA ($p = 0.04$) (Fig. 7, *middle three bars*) and loss of cell volume (data not shown). There was no statistically significant difference between cells treated with a randomized oligonucleotide plus thapsigargin and with the 5′-Bcl-x AS oligonucleotide plus thapsigargin.

Despite the lack of induction of apoptotic markers in 5′-Bcl-x AS oligonucleotide-treated cells, the survival of the MCF-7 cells treated with the 5′-Bcl-x AS oligonucleotide alone was consistently reduced (Fig. 8, *left three bars*). Interestingly, the additional treatment with thapsigargin had only a minor effect, if any, on cell survival (Fig. 8, *three right bars*). The difference in cell death after treatment with Bcl-x AS alone or in combination with thapsigargin was not statistically significant.

The oligonucleotide-induced cell death without evidence of cell apoptosis was intriguing, raising a possibility that the apoptosis assay based on DNA fragmentation may be inadequate in detecting apoptosis in the MCF-7 cell line. Therefore, we tested 5′-Bcl-x AS oligonucleotide-treated PC-3 and MCF-7 cells for PARP cleavage (Fig. 9), another indicator of apoptosis. Twenty-four hours after a single treatment with the 5′-Bcl-x AS oligonucleotide (0.08 μM for PC-3 cells and 0.4 μM for MCF-7 cells), this assay detected maximal apoptotic response of PC-3 cells, but virtually no apoptosis of MCF-7 cells. PARP cleavage did appear in MCF-7 cells at 48 h and reached maximum levels at 72 h post-treatment. However, even at the optimal time points, apoptosis of PC-3 cells was 3.5-fold higher than that of MCF-7 cells.

Factors Affecting Apoptosis of MCF-7 Cells—To gain some insight into the mechanisms responsible for the differences in the 5′-Bcl-x AS effects on PC-3 and MCF-7 cells, we compared the two cell lines for the levels of expression of Bcl-x_L, Bcl-x_S, and Bcl-2 proteins. Fig. 10*A* shows that the level of Bcl-x_L in MCF-7 cells (*lanes 4–6*) was markedly lower than that in PC-3 cells (*lanes 1–3*). This suggests that even if the same Bcl-x_L/Bcl-x_S mRNA ratio is induced by the oligonucleotide in the two cell lines, the absolute amount of generated Bcl-x_S protein will be lower in MCF-7 cells. Fig. 10*B* (*lane 5 versus 2*) illustrates this for treatment of PC-3 and MCF-7 cells with 0.08 and 0.4 μM 5′-Bcl-x AS, respectively. This treatment resulted in similar ratios of the splice variant mRNAs (data not shown). Note that the low level of Bcl-x_S protein in oligonucleotide-treated MCF-7 cells will also result in a low ratio of this protein to other anti-apoptotic gene products.

Since a wide variety of human cancers express not only Bcl-x_L, but also Bcl-2, a potent anti-apoptotic protein whose function is antagonized by Bcl-x_S (13), the expression of Bcl-2 was assayed in the two cell lines by immunoblotting of total protein. Densitometry of the immunoblot shown in Fig. 10*C* and quantitation of the results indicated that, in MCF-7 cells, the concentration of Bcl-2 was ~1.7 times higher than that in PC-3 cells (compare *lanes 1–3 versus 4–6*). As expected, the level of Bcl-2 was not affected by treatment of the cells with the 5′-Bcl-x AS or randomized oligonucleotide (Fig. 10*C*, *lanes 2* and *5* and *lanes 3* and *6*, respectively).

We have cultured MCF-7 cells in charcoal-stripped medium, a procedure shown to remove estrogen from the medium and, as a consequence, to inhibit expression of Bcl-2 in these cells (28). Under these conditions, the cellular level of Bcl-2 in MCF-7 cells was reduced below the level detected in PC-3 cells (data not shown). However, the treatment had no effect on apoptosis of 5′-Bcl-x AS oligonucleotide-treated MCF-7 cells (Fig. 7, *right three bars*). Thus, the contribution of Bcl-2 to the induction or the lack of apoptosis in 5′-Bcl-x AS oligonucleotide-treated cells remains unclear.

DISCUSSION

We have taken advantage of the fact that alternative splicing of Bcl-x pre-mRNA yields two products with antagonistic functions and used an oligonucleotide antisense to the 5′-splice site

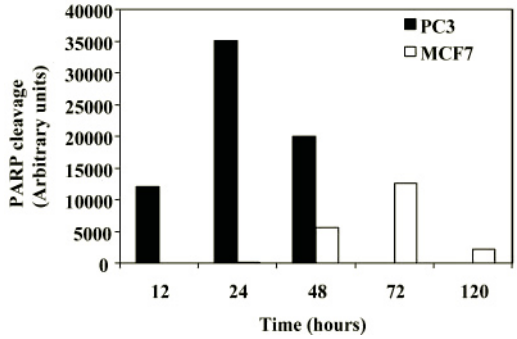

FIG. 9. **PARP cleavage in PC-3 and MCF-7 cells treated with 5′-Bcl-x AS.** Total protein from 5′-Bcl-x AS oligonucleotide-treated cells was analyzed by immunoblotting with anti-PARP antibody. The protein samples were collected at 12, 24, and 48 h for PC-3 cells and at 24, 48, 72, and 120 h for MCF-7 cells after oligonucleotide treatment. Band intensities were quantitated, and the results are expressed as the level of cleavage of PARP above background generated by treatment with the randomized oligonucleotide. *Black bars*, PC-3 cells (0.08 μM 5′-Bcl-x AS); *white bars*, MCF-7 cells (0.4 μM 5′-Bcl-x AS).

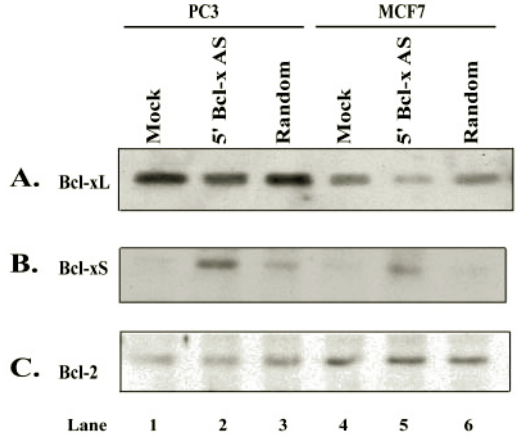

FIG. 10. **Expression of Bcl-x_L, Bcl-x_S, and Bcl-2 proteins in PC-3 and MCF-7 cells.** Total protein from PC-3 and MCF-7 cells was analyzed by Western blotting for Bcl-x_L (*A*), Bcl-x_S (*B*), and Bcl-2 (*C*) proteins. *Lanes 1–3*, PC-3 cells subjected to mock, 5′-Bcl-x AS (0.08 μM, 36 h), and randomized oligonucleotide (0.08 μM, 36 h) treatment, respectively; *lanes 4–6*, analogous treatment (0.4 μM, 36 h) of MCF-7 cells.

of Bcl-x_L to shift splicing from the anti-apoptotic splice variant, Bcl-x_L, to the pro-apoptotic splice variant, Bcl-x_S. This approach should be superior to antisense down-regulation of Bcl-x_L mRNA (39, 40) since, by definition, a decrease in Bcl-x_L leads to a concomitant increase in the concentration of the antagonistic Bcl-x_S, amplifying the biological effects of the treatment. In fact, an oligonucleotide targeted to the 5′-splice site of Bcl-x pre-mRNA, which led to an increase in the Bcl-x_S splice variant and a decrease in the Bcl-x_L splice variant, was a better inducer of apoptosis in PC-3 cells than an oligonucleotide targeted to the 3′-splice site, which decreased the expression of the Bcl-x_L protein.

Treatment of PC-3 and MCF-7 cells with the 5′-Bcl-x AS oligonucleotide led to a dose- and time-dependent shift in splicing of Bcl-x pre-mRNA from the Bcl-x_L to Bcl-x_S pathway and to a concomitant increase in the level of Bcl-x_S protein. However, even though most of the Bcl-x_L mRNA disappeared, the Bcl-x_L protein remained in the cells in apparent excess over the Bcl-x_S variant. There may be two reasons for this unexpected observation. First, the anti-Bcl-x antibodies may preferentially recognize the longer Bcl-x_L polypeptide and under-represent the Bcl-x_S splice variant. This possibility seems likely since several anti-Bcl-x antibodies from different manufacturers failed to detect the Bcl-x_S polypeptide altogether. Second, the Bcl-x_L protein may be very stable, persisting in the cells at high concentrations even though its *de novo* translation was markedly reduced. The latter interpretation suggests that, especially in PC-3 cells, Bcl-x_S has *trans*-dominant properties, promoting apoptosis and/or cell death despite the existing excess of anti-apoptotic Bcl-x_L. Since Bcl-x_S binds to Bcl-x_L and inhibits its anti-apoptotic action (20), one has to conclude either that the binding is not stoichiometric or that this is not the only mechanism responsible for the pro-apoptotic properties of Bcl-x_S (20).

It appears that the minimal apoptotic response of MCF-7 cells to oligonucleotide treatment is mostly due to the low level of Bcl-x_S protein generated by the shift in the Bcl-x_L/Bcl-x_S mRNA ratio. It follows that the ratio of Bcl-x_S protein to other apoptotic regulatory proteins that may impact its function may also be lower. However, the identity and nature of the genes that may be responsible for the apoptotic resistance of MCF-7 cells are not clear and require additional studies.

For instance, a shift in alternative splicing of Bcl-x pre-mRNA was also observed in another breast cancer cell line, HS578T,[2] and, while this work was in progress, in a lung adenocarcinoma cell line, A549, treated with 2′-*O*-methoxyethoxy-modified oligoribonucleotides (41). Among these cell lines, as well as the ones investigated here, the *p53* gene is mutated in PC-3 (42, 43) and HS578T (44) cells, but not in A549 (41) or MCF-7 (44) cells, and Bcl-2 is low in PC-3 (Fig. 10*C*) (8), HS578T (44), and A549 (41) cells and high in MCF-7 cells (Fig. 10*C*) (44). Yet treatment with 5′-Bcl-x AS alone led to significant apoptosis only in PC-3 cells, whereas a decrease in Bcl-2 in MCF-7 cells to levels below those in PC-3 cells did not promote oligonucleotide-induced apoptosis. It appears that if the *p53* and *bcl-2* genes do play a role in modulating the function of the 5′-Bcl-x AS oligonucleotide, their effects may depend on additional cellular background. In this regard, it is notable that Bcl-x_L was found in 100% of prostate adenocarcinomas, but Bcl-2 was found in only 25% (19). However, in some breast carcinomas, as well as in some breast cancer cell lines, the levels of Bcl-2 were higher than those of Bcl-x_L (45).

The different responses of PC-3 and MCF-7 cells to the shift in Bcl-x pre-mRNA splicing are not limited to generation of subdiploid DNA, but also to cleavage of PARP, a different apoptotic marker, and to cell death assayed by colony formation. In this regard, 5′-Bcl-x AS promoted cell death in both PC-3 and MCF-7 cell lines, further complicating the interpretation of the results. Approximately 39% of MCF-7 cells and 24% of PC-3 cells survived the treatment with 5′-Bcl-x AS (0.4 μM oligonucleotide for MCF-7 cells and 0.08 μM for PC-3 cells) that resulted in a similar ratio of Bcl-x_L to Bcl-x_S mRNA. Recalculation of the data in terms of the effective concentration (EC_{50}) of the oligonucleotide yielded EC_{50} values of 0.32 and 0.05 μM for MCF-7 and PC-3 cells, respectively, a 6.5-fold ratio. This difference is partly explained by reduced nuclear uptake of the oligonucleotide in MCF-7 cells. In contrast to PC-3 cells, in which fluorescent labeled oligonucleotide accumulated predominantly in the nucleus, in MCF-7 cells, the large fraction of the compound remained concentrated in cytoplasmic endosomal vesicles (data not shown), where it was unable to affect splicing, a nuclear process (46).

The fact that cells differ in their response to a shift in the Bcl-x_L/Bcl-x_S ratio may impart beneficial specificity to the *in vivo* applications of the 5′-Bcl-x AS oligonucleotide. That is, although Bcl-x_L is expressed in a number of cell types, including several hematopoietic cell lineages (47), the apoptotic stim-

[2] D. R. Mercatante and R. Kole, unpublished data.

ulus of the oligonucleotide may be effective only in certain susceptible cells with a gene expression profile akin to the prostate cancer PC-3 cell line. In addition, a combination of the oligonucleotide with chemotherapeutic agents exemplified by cisplatin in A549 cells (41) may sensitize only certain types of cells to undergo apoptosis. An additional benefit of cellular sensitization by the oligonucleotide may be a reduction in the dosage of chemotherapeutic agents and hence in the overall toxicity of cancer treatment.

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