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Increased Bcl2 Expression by Antisense Oligoribonucleotides Targeting the Adenine-Uridine-Rich Element Motif

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ABSTRACT

RNA has become a promising target for pharmacological purposes. Most current strategies are directed toward down-regulating its functions. In this study, we provide evidence of the up-regulation of messenger RNA in a sequence-specific manner. The bcl2 (b)-ARE (adenine-uridine-rich element) in the 3′-untranslated region of the b-RNA that regulates the rate of RNA degradation has been targeted with three chemically modified oligoribonucleotides designed in the antisense orientation (asORNs). The three asORNs were studied by a cell-free degradation assay. All three slowed the rate of RNA decay in a dose-response fashion, they were specific to the b-ARE, and two of them were individually effective. asORNs were then transfected into the malignant cells in culture and b-RNA half-life was measured by real-time reverse transcriptase-polymerase chain reaction. We showed that by stabilizing b-RNA the three asORNs increased the expression of b-RNA and of the relevant protein in a dose-response fashion.

The RNA molecule has a pervasive role in contemporary biology, especially with regard to the most fundamental and highly conserved cellular processes (Joyce, 2002). A major challenge has been to unravel how RNA controls its maturation and translation activities. Understanding these basic mechanisms can lead to effective ways of controlling gene expression artificially.

A first generation of compounds designed to control gene expression by targeting RNA is represented by the antisense oligodeoxynucleotides (Scherer and Rossi, 2003). The strategy has potential in studies of cell biology (Ravichandran et al., 2004) and for clinical applications (Kalota et al., 2004); antisenses have served to disclose the simplicity of the system and the specificity of action (Schiavone et al., 2004).

Most exciting is RNA silencing, a new field of research that has coalesced during the last decade from independent studies on various organisms (Meister and Tuschl, 2004). The regulatory importance of small noncoding RNA is now recognized, and their mechanism of action is increasingly understood (reviewed in Gottesman, 2004; Storz et al., 2004).

The laboratory exploitation of most of these RNA molecules is focused on decreasing or knocking out gene expression. In contrast with these approaches, we have found that RNAs can be specifically up-regulated, targeting certain motifs such as the degradation elements in *cis*. In fact, a major control point in gene expression is the turnover of mRNA (Wilusz and Wilusz, 2004).

It is now well established that mRNA decay is not a default process but a tightly regulated process dependent on specific *cis*-acting sequences and *trans*-acting factors (Bevilacqua et al., 2003b). An important example of a specific *cis*-acting element controlling the half-life of mRNA is the adenine-uridine-rich element (ARE) found mainly in the 3′-untranslated regions (UTRs) of many unstable mammalian mRNAs. So far, it represents the most widespread and efficient determinant of RNA (in)stability among those characterized in mammalian cells (Shaw and Kamen, 1986).

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ABBREVIATIONS: ARE, adenine-uridine-rich element, b, bcl2; b-RNA, 3′-untranslated region of bcl2 mRNA; UTR, untranslated region; ORNs, 2′-*O*-methyl oligoribonucleotides; asORNs, 2′-*O*-methyl oligoribonucleotides in antisense orientation; degORNs, 2′-*O*-methyl degenerated oligoribonucleotides; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; AUBP, ARE-binding protein; RT, reverse transcriptase; IGFR, insulin-like growth factor receptor; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate.

In this study, we focused on the bcl2 mRNA (b-RNA) that codes for an antiapoptotic protein involved in neoplastic transformation and/or progression. Bcl2 protein has been shown to lower cell sensitivity to chemotherapy and radiotherapy (Reed et al., 1996; Kroemer, 1997), whereas reduced *bcl2* expression has been associated with degenerative disorders, including Parkinson's and Alzheimer's diseases (Saille et al., 1999). In addition to sparing neurons from degeneration (Chen et al., 1997), the rate of bcl2 mRNA degradation might play a role in synapse metabolism.

A further inspiration for these studies came from the discovery of an antisense RNA complementary to b-ARE, which induces overexpression of the Bcl2 protein in the human follicular B-cell lymphoma (Capaccioli et al., 1996).

In previous studies, we described an ARE domain located in the 3′-UTR of b-RNA (b-ARE) that is required for b-RNA decay in vivo (Morelli et al., 1997). We have also shown that the activation of an apoptotic program specifically induces an ARE-dependent b-RNA degradation (Schiavone et al., 2000) and is accompanied by modifications of proteins binding the b-ARE (Donnini et al., 2001).

In the work described here, we used pharmacological tools to inactivate the b-ARE motif within the b-RNA, thereby stabilizing b-RNA and consequently increasing the concentration of the Bcl2 protein in the cells. In particular, we designed 2′-*O*-methyl oligoribonucleotides (ORNs) in the antisense orientation to b-ARE and used them in an in vitro degradation assay as well as in cultured cells to protect b-RNA from degradation. We showed that antisense ORNs (asORNs) complementary to a critical *cis*-acting element for b-RNA stability can specifically up-regulate the expression of Bcl2 protein.

Materials and Methods

Cell Cultures and Chemicals. The cell lines, human follicular t(14,18)-positive B-cell lymphomas DOHH2, promyelocytic leukemia HL60, and neuroblastoma SHSY-5Y, were maintained in RPMI 1640 medium supplemented with 1% glutamine, antibiotics, and 10% fetal calf serum in a humidified atmosphere of 5% CO_2 at 37°C. Viability was monitored by the trypan blue exclusion assay. Three 2′-*O*-methyl antisense oligoribonucleotides were provided by Dharmacon, Inc. (Lafayette, CO), having been designed based on the ARE core sequence of *bcl-2* gene as shown in Fig. 1; a 26-mer degenerated ORN (degORN), 5′-NNNNNNNNNNNNNNNNNNNNNNNNNN-3′ (where N is A, G, C, or U), was used as control. The transcription blocker 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was purchased from Sigma-Aldrich (St. Louis, MO).

Plasmids and In Vitro Transcription. 3′-UTR fragments of bcl-2 mRNA obtained by PCR were cloned in a transcription vector and used for in vitro synthesis of transcripts using SP6 RNA polymerase according to the manufacturer's instructions (Promega, Charbonnières, France), incorporating digoxigenin-labeled NTPs (Roche Diagnostics, Basel, Switzerland) as described previously (Bevilacqua et al., 2003a).

Cell-Free RNA Degradation System and Assays of Inhibition by ORNs. Cell-free RNA degradation assays were performed as described by Bevilacqua et al. (2003a) with minor modifications. Ten micrograms of carrier tRNA and 5 ng of digoxigenin-labeled b-RNA and IGFR-RNA were incubated for 5 min at 80°C after 10 min at 51°C with unlabeled ORNs at the indicated doses before the incubation with cell extracts at 37°C. The relative amount of labeled RNAs was determined by densitometric analysis.

ORN Transfection. AsORNs and degORN at the concentrations described in the text have been delivered with an equal volume of DOTAP Liposomal Transfection Reagent (Roche Diagnostics) to 7.5×10^5 cells/ml according to the manufacturer's instructions.

Measurement of b-RNA Half-Life. At day 1 after ORN transfection, 7.5×10^5 cells/ml were treated with the transcription blocker DRB at a final concentration of 20 μg/ml. At 0, 3, 5, and 7 h after DRB addition, 2×10^6 cells were collected and RNA was extracted for further analysis.

Total RNA Extraction and Real-Time RT-PCR. Total cellular RNA was extracted at the indicated times using the NucleoSpin RNA II columns (Macherey-Nagel, Dueren, Germany) according to the manufacturer's instructions. The RNA was treated with RNase-free DNase (Invitrogen, Carlsbad, CA) and analyzed spectroscopically and by gel electrophoresis for purity and integrity, respectively. For synthesis of cDNA, 0.1 μg/μl total RNA was reverse-transcribed with High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) using standard conditions set by the manufacturer in a total volume of 50 μl. Levels of bcl2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs of each sample were determined by quantitative real-time PCR after TaqMan Universal MasterMix (Applied Biosystems) standard conditions set by the manufacturer; 5 μl of total cDNA was amplified with 2× TaqMan Universal MasterMix Buffer, 20× predeveloped assay reagents system-targeted bcl2, 20× predeveloped assay reagents system control GAPDH, and nuclease-free water (Promega) to a total volume of 25 μl. Each reaction was performed in triplicate for a better statistical reliability of results. The PCR reactions were carried out in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) under the following conditions: 50°C for 2 min, 95°C for 10 min, 95°C for 15 s, and 1 min at 60°C (40 cycles). The amplification plot and cycle threshold data were elaborated with ABI Prism 7000 SDS software (version 1.1). GAPDH data were used to normalize bcl2 cDNA values.

Western Blot Analysis. The assay was performed under standard conditions. In brief, cells were washed twice in ice-cold phosphate-buffered saline and resuspended in 100 μl of ice-cold radioimmune precipitation assay lysis buffer, vortexed for 3 s, and incubated on ice for 30 min. The lysates were centrifuged at high speed for 20 min at 4°C. Equal amounts of proteins were analyzed by 12% SDS-PAGE, blotted onto nitrocellulose membrane Hybond-enhanced chemiluminescence (ECL; Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) in a Bio-Rad Trans-blot apparatus at 100 V for 90 min. Blots were processed by an ECL Plus detection kit as instructed by the supplier (Amersham Biosciences). The blots were probed with a mouse anti-Bcl-2 (DakoCytomation Denmark A/S, Glostrup, Denmark) and a rabbit anti-actin (Sigma-Aldrich)

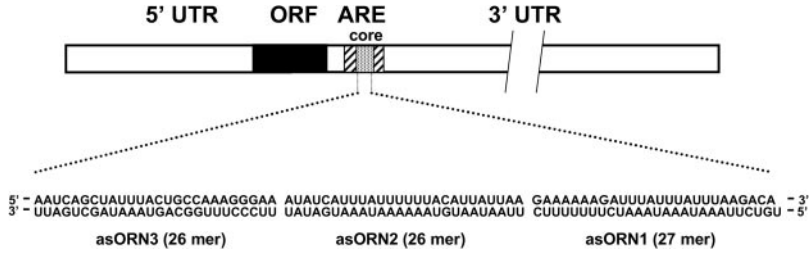

Fig. 1. Schematic structure of b-RNA, core b-ARE nucleotide sequence, and complementary asORNs. Human b-ARE sequence (bases 942-1020, GenBank accession number M14745) and three complementary 2′-*O*-methyl-modified asORNs are depicted.

antibody followed by a horseradish peroxidase-conjugated secondary antibody.

Results

The ARE motif located in the 3′-UTR of b-RNA was targeted with three sequential asORNs. First, we explored the possibility of targeting a regulative sequence inside an mRNA without affecting its ability to be translated. A further goal was to investigate whether the inhibition of the b-ARE would up-regulate the amount of the b-RNA and the relevant protein in growing cells. This is not a trivial goal, because modalities to increase the expression of a single gene by exogenous means are not actually available. In this study, were used the 2′-*O*-methyl derivatives because they are nuclease-resistant but have full hybridizing capacity. Three asORNs, each complementary to one third of the b-ARE motif (Fig. 1), were used in vitro or transported into the cells by liposomal transfection.

Inhibition by ORNs of b-ARE Degradation in Cell-Free Assays. The effect of asORNs on the rate of b-ARE degradation was studied in a cell-free system. Transcripts were neither capped at the 5′ end with ^{7}mGpppG nor polyadenylated at the 3′ end, because ARE-directed degradation of an mRNA can occur in a cap-independent fashion (Brewer, 1998; Detich et al., 2001) and in the absence of a poly(A) tract (Chen et al., 2001; Lai and Blackshear, 2001). For assay of decay times, unfractionated cell lysates derived from exponentially growing DOHH2 human lymphoma cells were mixed with the relevant transcripts and incubated at 37°C for various times as described previously (Bevilacqua et al., 2003a).

The rate of b-ARE degradation was significantly reduced by the addition of the asORNs to the degradation mixture, as shown in Fig. 2A. The asORNs exhibited their stabilizing activity selectively on b-RNA and not upon the IGFR control transcript. However, the addition of fully degenerated ORNs (degORNs) did not significantly change the 30-min half-life of b-ARE incubated with cell extracts (Fig. 2B). The half-life of b-ARE was increased to more than 60-min in samples containing the asORNs. Moreover, the inhibition of b-ARE turnover by the asORNs was specific and dose-dependent from 1 to 30 μM (Fig. 2C). The activity of individual asORNs was analyzed at 10 μM under the conditions described above. AsORN2 was the most effective (Fig. 3B).

Stabilization and Overexpression of b-RNA in Two Cell Lines. The turnover of cellular b-RNA exposed to asORN was studied by real-time reverse transcriptase (RT)-PCR (TaqMan probe system) in SHSY-5Y and HL60 cell lines, a neuroblastoma growing in adhesion, and a leukemia cell line growing in suspension.

SHSY-5Y cells were transfected with a 500 nM concentration of each of the three asORNs. At different times, total RNA was extracted from the cells and amplified by real-time RT-PCR. The mixture of the three asORNs at a total dose of 1.5 μM significantly increased the total amount of b-RNA in the SHSY-5Y cells (Fig. 4A).

HL60 cells receiving the three asORNs for 24 h were then treated with 20 μg/ml DRB to inhibit transcription. At different times after DRB addition, total RNA was extracted and amplified. Figure 4B shows that asORNs slowed the rate of degradation in DRB-treated cells. In cells treated with degORNs, the half-life of b-RNA was approximately 3 h, which was almost doubled in the asORN-treated cells. It is worth noting that primers for RT-PCR amplification were taken from the coding region of the b-RNA.

Up-Regulation of Bcl2 Protein in Two Cell Lines. The neuroblastoid SHSY-5Y cells were transfected with the three asORNs by DOTAP. The Bcl2 protein was quantified after 5 days of treatment and compared with the amount of Bcl2 in untreated cells and in cells treated with degORNs (Fig. 5A); asORNs induced a 2.5-fold increase in the amount of Bcl2.

Figure 5 also shows the dose-response histograms of asORNs action on Bcl2 protein levels. Bcl2 protein was overexpressed after exposure to asORNs of both the myeloid HL60 (Fig. 5C) and especially the neuroblastoid SHSY-5Y (Fig. 5B) cell lines. Different accumulation rates might depend on the molecular milieu at the level of the decay machinery with which asORNs are considered to interfere. It is noteworthy that a higher rate of degradation has been shown in cell lines that express a higher level of *bcl2* (Bevilacqua et al., 2003a). On the other hand, a higher transcription rate of the *bcl2* gene could account for a more rapid accumulation. The most effective dose was 1 μM given at day 0 and at day 3. No obvious toxic effects have been observed.

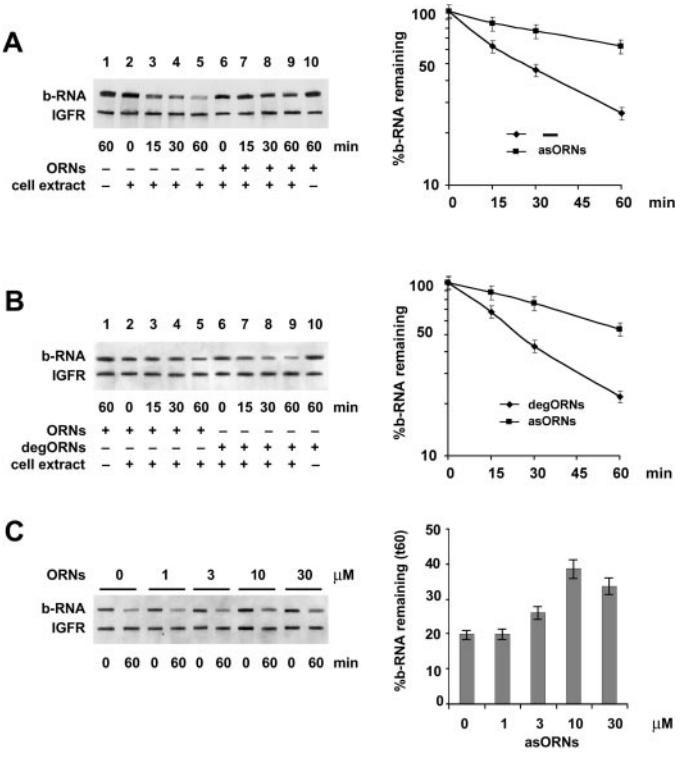

Fig. 2. In vitro b-RNA degradation assays. A, left, three asORNs (total dose, 10 μM) were added to the b-RNA and IGFR-RNA degradation mixture. Lane 1, synthetic transcripts incubated for 60 min without cell extract and without asORNs; lanes 2 to 5, transcripts incubated with cell extracts for the indicated times; lanes 6 to 9, transcripts incubated with cell extract and asORNs for the indicated times; lane 10, transcripts incubated for 60 min without cell extract and with asORNs. Right, time course of decay of b-RNA from the left. B, left, lane 1, synthetic transcripts incubated for 60 min without cell extract and with asORNs; lanes 2 to 5, asORNs; lanes 5 to 9, degORNs. Right, time course of decay of the transcripts from the left; lane 10, transcripts incubated for 60 min without cell extract and with degORNs. C, left, assay conditions as in A. Doses of asORNs added to each tube are indicated. Right, histogram of the dose activity from the left (mean values) obtained by scanning laser densitometry from three assays. Bars indicate the mean ± S.E.

This work supports the concept that inhibition of b-RNA turnover can increase the expression of the relevant gene at the phenotypic level. It also shows that the inhibition of a subregion within a messenger need not inhibit the activity of other domains.

Discussion

The concentration of mRNA in cells is a major determinant of the amount of protein expression and phenotype. Although most of the emphasis in the regulation of RNA levels in cells has been placed on transcription, the rate of RNA degradation has recently assumed greater importance (Wilusz and Wilusz, 2004). Fine-tuning of the levels of RNA in the cells through the rapid degradation of redundant products can be achieved by a number of mechanisms (Meyer et al., 2004). *cis*-Elements can regulate the RNA level at the steady state and, most relevant, "on demand" by particular metabolic conditions or conditions of stress (Kaempfer, 2003).

Identification of the biochemical machinery for RNA degradation has introduced new paradigms and tools of great value for the application in therapeutic pharmacology. RNA displays the unique properties because of its nucleotide sequence, and it can be easily targeted in the cytoplasm.

The work discussed here focuses on the up-regulation of gene expression by inhibition of mRNA decay. The target is the *cis*-element b-ARE in the 3′-UTR region of the b-RNA. The great sensitivity of b-ARE to physiological or pathological conditions (Lapucci et al., 2002) makes b-ARE an ideal target for regulation of gene expression. Indeed, the last of the findings reported provides experimental evidence that b-RNA degradation can be regulated specifically despite the huge number of different RNA molecules expressed in the cytoplasm.

In this study, we have used exogenous means to inhibit b-ARE activity to slow down the rate of b-RNA degradation and possibly to increase the amount of b-RNA. The final goal, namely the up-regulation of Bcl2 protein, might also be obtained by acting at the level of ARE-binding proteins (AUBPs) that form part of the degradation complex (Raineri et al., 2004). However, these AUBPs recognize a whole set of genes and are thus not specific to a single gene (Chen et al., 2001).

The 2′-*O*-Me ORNs are believed not to trigger cellular reactions unless properly synthesized to degrade RNA through a small interfering RNA mechanism. It is conceivable that they might hybridize a whole stretch of RNA, causing the temporary inhibition of its functions, possibly through steric hindrance. Therefore, we prepared synthetic ORNs in antisense orientation to b-ARE to decrease the rate of b-RNA degradation.

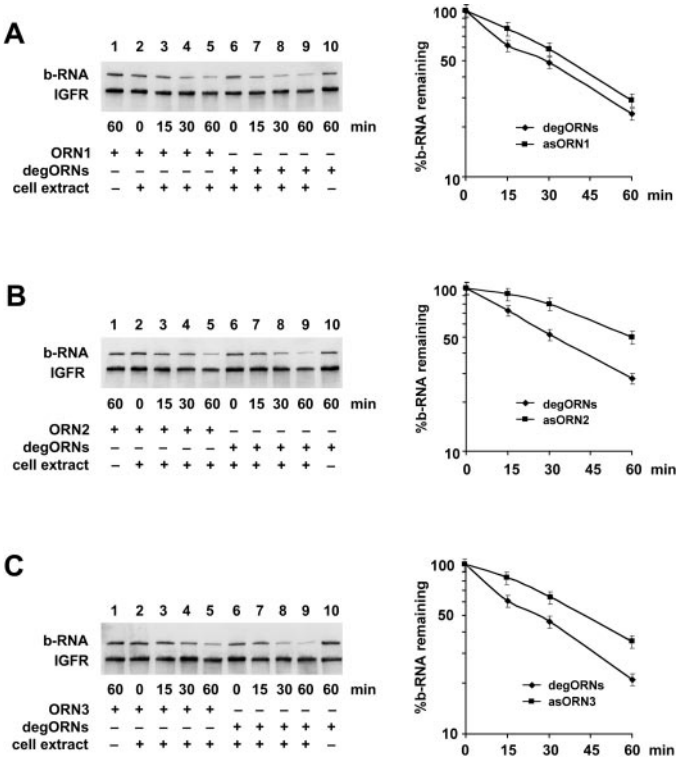

Fig. 3. Inhibition of b-RNA degradation by individual asORNs. Left, assay conditions as in Fig. 2A. asORN 1 was added to reaction mixture in A, asORN2 was added to reaction mixture in B, asORN3 was added to reaction mixture in C at a dose of 10 μM. Right, time course of decay from the left.

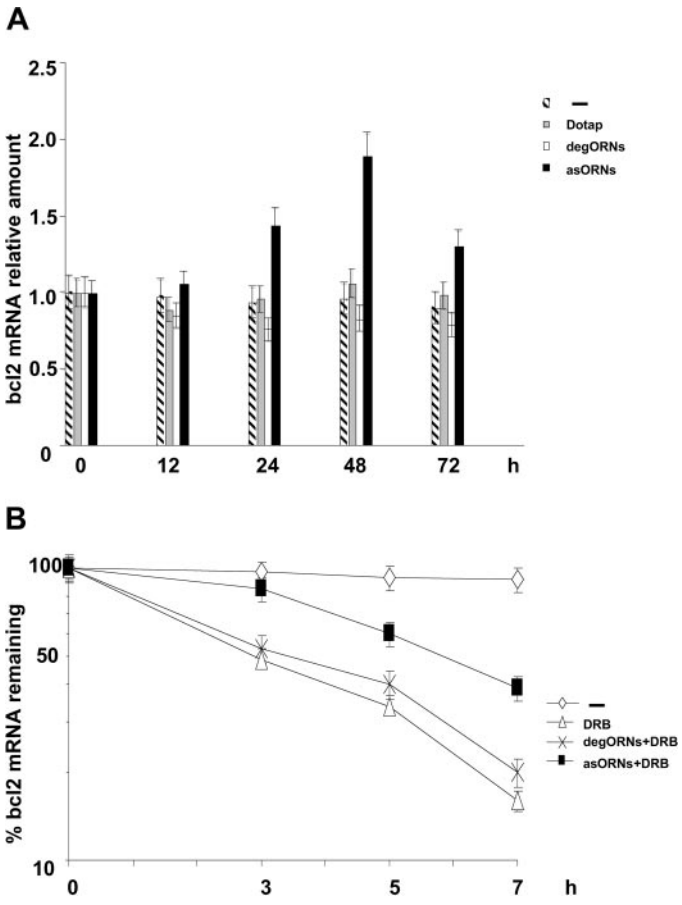

Fig. 4. b-RNA determination in cells treated with asORNs. A, to a final concentration of 500 nM each, asORNs were delivered with DOTAP liposomal transfection reagent into SHSY-5Y cells. To a final concentration of 1.5 μM, degORNs were given under the same conditions. Cells were untreated or exposed to DOTAP as further controls. RNA obtained from 2×10^6 cells at the indicated times after transfection was analyzed by quantitative real-time RT-PCR. Concentrations of bcl2 were normalized with GAPDH cDNAs. Means ± S.D. from three independent experiments in triplicate are indicated. B, HL60 cells transfected with asORNs as described in A were treated with the transcription inhibitor DRB (20 μg/ml). At the indicated times, b-RNA was analyzed by quantitative RT-PCR and normalized with GAPDH cDNAs. Means ± S.D. from three independent experiments in triplicate are indicated.

In a cell-free degradation system, the three designed asORNs significantly slowed the rate of b-RNA degradation. The activity was restricted to b-RNA and was dose-dependent. asORN2 and asORN3 were significantly effective but less potent than when acting together.

The asORN activities were studied in two cell lines transfected with a 500 nM concentration of each asORN. The rate of b-RNA degradation was significantly reduced, as revealed by real-time RT-PCR assay. asORNs doubled the half-life of b-RNA in cells from 3 to 6 h. In a further set of studies, asORNs increased above the basal level, the total amount of b-RNA in the cells. Thus, b-ARE is a suitable target to increase RNA expression in a gene-specific way.

The last set of studies in both HL60 and SHSY-5Y cell lines was carried out to establish whether asORNs are able to up-regulate Bcl2 protein. Although protein amount is finely regulated within cell, asORNs hindering the binding site of degradation promoting AUBPs on b-RNA were able to more than double the amount of Bcl2 protein compared with cells transfected with control degORNs.

All of these findings strengthen the prominent role of AU-rich elements in mRNA decay and depict a new strategy of increasing the expression of a specific gene by acting at the level of a degradation element. The amount of Bcl2 protein was more than doubled relative to the cells transfected with control degORNs. These findings all support the new strategy of increasing the expression of a specific gene by acting at the level of a degradation element. The possibility of up-regulating the concentration of a single protein will surely find therapeutic applications in a variety of diseases or physiological conditions, including those resulting from insufficient production of important factors or abnormal degradation of a particular RNA (Roos et al., 2004).

It is interesting that apoptotic neurons associated with

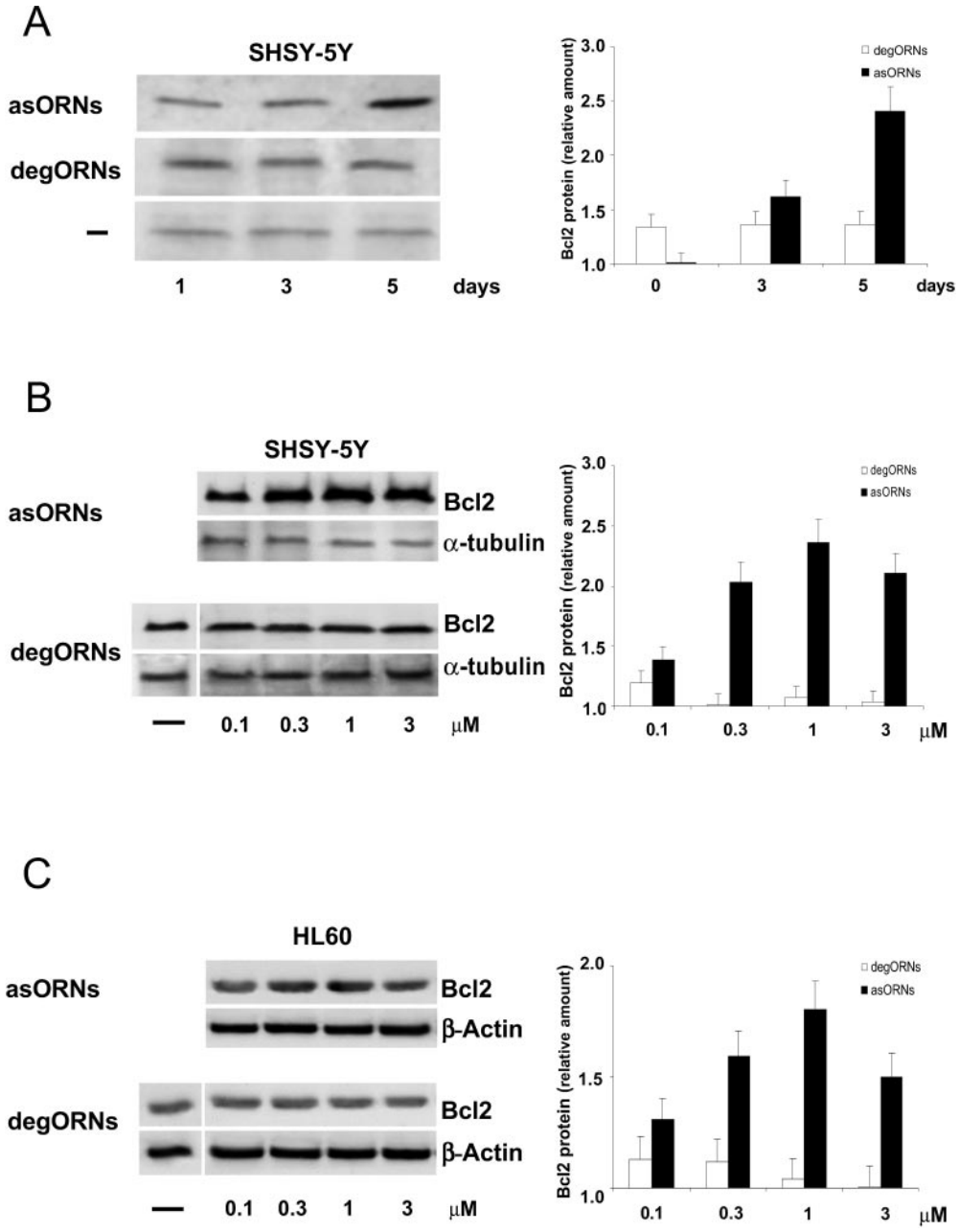

Fig. 5. Bcl2 protein determinations in cells treated with asORNs. A, asORNs and degORNs were delivered—1.5 μM at day 0 and 0.75 μM at day 3—into SHSY-5Y cells in the same conditions as in Fig. 4A. Left, cell samples were analyzed for Bcl2 protein levels by Western blot assay on days 0, 3, and 5. Right, densitometric histograms of relative band intensities from three independent Western blot experiments. B and C, asORNs and degORNs at the indicated concentrations were given to SHSY-5Y cells (B) or HL60 cells (C) on day 0 and halved on day 3. Left, Western blot of Bcl2 protein on day 5, normalized with β-actin in HL60 cells and normalized with α-tubulin in SHSY-5Y cells. Right, densitometric histograms of relative band intensities from three independent Western blot experiments.

neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases, have altered Bcl2 expression (Vyas et al., 1997; Mattson, 2000). Actually Bcl2, in addition to its anti-apoptotic functions, has shown differentiative and neurotrophic properties. It can promote dendritic branching and produce regeneration of damaged neurons (Chen et al., 1997). Therefore, Bcl-2 up-regulation represents a strategic goal in neuroprotection therapies (Mattson, 2000).

Such regulation can be achieved by exogenous means as shown here or by transfecting cells with a segment of gene expressing an RNA in antisense orientation as occurring in a human lymphoma and in development (Lee and Roth, 2003; Friedrich et al., 2004). The simplicity of the approach opens new avenues for the regulation of gene expression, because ARE-mRNAs encode a wide range of functionally diverse proteins that belong to different biological processes and are important in several disease states (Khabar, 2005). Although the use of synthetic ORNs may seem to have limited potential in clinical application because ORNs do not cross cell membranes, many efforts are actually underway to overcome this limitation (Soutschek et al., 2004).

Acknowledgments

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