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Stabilization of Cellular mRNAs and Up-Regulation of Proteins by Oligoribonucleotides Homologous to the Bcl2 Adenine-Uridine Rich Element Motif[S]

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ABSTRACT

Adenine-uridine rich elements (AREs) play an important role in modulating mRNA stability, being the target site of many ARE-binding proteins (AUBPs) that are involved in the decay process. Three 26-mer 2′-*O*-methyl oligoribonucleotides (ORNs) homologous to the core region of ARE of bcl2 mRNA have been studied for decoy-aptamer activity in UV cross-linking assays. Sense-oriented ORNs competed with the ARE motif for the interaction with both destabilizing and stabilizing AUBPs in cell-free systems and in cell lines. Moreover, ORNs induced mRNA stabilization and up-regulated both Bcl2 mRNA and protein levels in the cells. Bcl2 ORNs stabilized other ARE-containing transcripts and up-regulated their expression. These results indicate that Bcl2 ORNs compete for AUBP-ARE interactions independently of ARE class and suggest that in the cell, the default labile status of ARE-containing mRNAs depends on the combined interaction of such transcripts with destabilizing AUBPs.

In mammalian cells, each step in the pathway of gene expression, from DNA to protein, is efficiently regulated. Although gene expression depends primarily on transcription, mRNA is regulated by various post-transcriptional events (Kindler et al., 2005; Moore, 2005; Riddihough, 2005). After the relatively slow process of transcription, the basal level of mRNA is mainly determined by the rate of degradation (Wagner and Lykke-Andersen, 2002; Cougot et al., 2004). Many messengers are further fine-tuned in response to environmental and physiological conditions (Shim and Karin, 2002).

Most inducible genes, including those coding for growth factors, receptors, or regulatory proteins, have unstable mRNAs because cells may have to modulate the corresponding protein level rapidly (Chen and Shyu, 1995). For these genes, the rate of mRNA turnover is the crucial step in regulating basic functions, including cell cycle, cell viability, and reaction to stress (Winzen et al., 2004; Cheadle et al., 2005).

The differences in phenotypes among cells are due mainly to untranslated sequences involved in regulation rather than in coding sequences. Regulatory factors include RNA binding proteins that heavily influence individual's proteome by regulating RNA stability and translation. These proteins associate with multiple messenger RNAs according to a model of coordinated gene expression of functionally related transcripts (Keene and Lager, 2005).

At the level of individual transcript, we have shown that the turnover of the bcl2 messenger can be regulated by the protein Bcl2 itself. A combinatorial mechanism is emerging of RNA regulation by multitarget proteins responsible for different levels of specificity and functionality.

Two major degradation mechanisms have been shown to affect mammalian mRNAs half-life: deadenylation decap-

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ABBREVIATIONS: ARE, adenine-uridine rich element; AUBP, adenine-uridine binding protein; ORN, oligoribonucleotide; UTR, untranslated region; b-ARE, bcl2 adenine-uridine rich element; b-RNA, 3′-untranslated region of Bcl2 RNA; HEK, human embryonic kidney; degORNs, 2′-*O*-methyl-degenerated oligoribonucleotide; DRB, 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole; GST, glutathione transferase; RT-PCR, reverse transcription polymerase chain reaction; MoAb, monoclonal antibody; IGFR, insulin-like growth factor-I receptor; nt, nucleotide MKK6, mitogen-activated protein kinase kinase 6; Rluc, *Renilla reniformis* luciferase.

ping-mediated decay (Gao et al., 2001; Bail and Kiledjian, 2006), and decay mediated by the ARE motives (Guhaniyogi and Brewer, 2001; Raijmakers et al., 2004). In both pathways, gene-specific stretches, usually located in the 3′-UTR and 5′-UTR, are needed for degradation (Stoecklin et al., 2006).

The AREs are discrete nucleotide structures located in the 3′-UTR, presently identified in more than 4000 inducible genes including cyclins, kinases, cytokines, receptors, stress genes, and proto-oncogenes (Bakheet et al., 2006). These elements have an important role in mRNA stability, being the target site of many AUBPs involved in the decay processes and being able to recruit the exosome (Bevilacqua et al., 2003b; Barreau et al., 2006).

mRNA turnover has been found to be located in discrete cytoplasmic foci, the processing bodies. Under stress conditions that lead to stalled translation, preinitiation complexes can aggregate in cytoplasmic granules from which selected transcripts are degraded (Brengues et al., 2005; Kedersha et al., 2005).

Understanding the molecular mechanisms that regulate the rate of degradation and learning how each mRNA is maintained at the desired level in the cellular cytoplasm are crucial issues. Future studies might unravel new pathogenic pathways leading to many diseases, including cancer (Audic and Hartley, 2004; Denkert et al., 2004) or neurodegenerative disorders (Si et al., 2004) and disclose new therapeutic strategies to regulate gene expression by exogenous means. Moreover, inhibition of the degradation process increases the cellular amount of RNA and of its relative protein.

The regulation of inducible proteins causes obvious phenotypes. For instance, increasing the cellular level of the anti-apoptotic protein Bcl2 can protect cells from apoptotic stimuli, although it might have transforming effects (Meijerink et al., 2005). The degradation of Bcl2 mRNA is an ARE-dependent process (Schiavone et al., 2000) mediated by *trans*-acting elements (Donnini et al., 2004). The rate of Bcl2 mRNA degradation is also dependent on the amount of the Bcl2 protein in the cells (Bevilacqua et al., 2003a).

In the present work, three ORNs designed on the ARE motif (b-ARE) of Bcl2 mRNA have been studied through UV

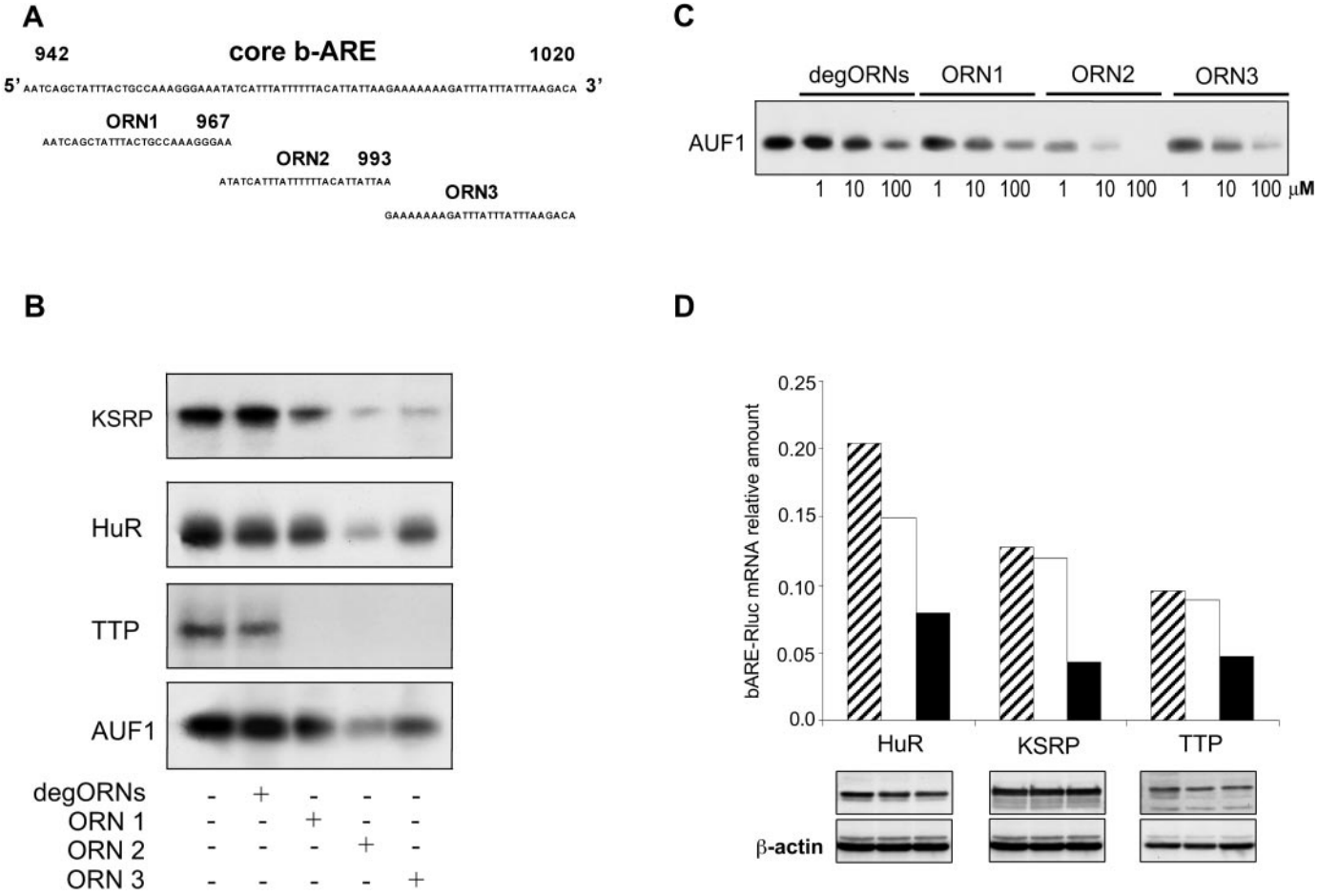

Fig. 1. ORN displacement of AUBPs from b-ARE. A, schematic structure of b-ARE and nucleotide positions from which ORNs were designed. Human b-ARE sequence (bases 942–1020, GenBank accession number M14745), and three homologous 2′-*O*-methyl-modified ORNs are depicted. B, competition by ORNs for AUBPs in the UV cross-linking assays. Radiolabeled b-RNA was incubated with the AUBPs KSRP, HuR, TTP, or AUF1. Before the UV exposure, degORNs or individual ORNs were added at 1 μM to the reaction mixture. C, dose-response relationship of ORNs. The displacement of AUF1 from the target b-ARE using increasing amounts of ORNs in the UV cross-linking assays is shown. D, RNA immunoprecipitation assays. Extracts from HEK 293 cells stably transfected with the chimeric Rluc-b-ARE construct and treated with 1.5 μM ORNs were immunoprecipitated with αHuR MoAb or αKSRP and αTTP polyclonal antibodies. The amount of Rluc-b-ARE in the HuR, KSRP, or TTP immunocomplex was evaluated by real-time PCR and normalized to input RNA of corresponding samples. ▨, untreated; □, degORNs; ■, sense ORNs. Equal amounts of HuR, KSRP, or TTP were detected in ORNs of treated and untreated samples, as shown in Western blots (bottom). The data are representative of one of three independent experiments.

cross-linking assays to prove their AUBP inactivation according to a decoy-aptamer mechanism.

Several studies indicate that AUBPs, including KSRP, TTP, AUF1, elicit a destabilizing activity, promoting the rapid decay of unstable mRNAs, although HuR may exhibit an opposite effect. Sense-oriented ORNs were used to compete for the binding of these AUBPs to synthetic b-ARE and were studied for their effects on the rate of ARE-dependent RNA decay. The rate of RNA degradation, the level of RNA expression, and the amount of protein synthesized were evaluated in cell lines exposed to ORNs. The present study aims at uncovering a new mechanism for the up-regulation of gene expression based on competition for AUBPs activity.

Materials and Methods

Cell Cultures and Chemicals. Viable human lymphoma cell lines DOHH2, HL60, and Raji in RPMI 1640 or viable monolayer HEK 293 cells were grown in complete Dulbecco's modified Eagle's medium, in combination with 10% fetal calf serum. Three 2′-*O*-methyl ORNs, designed on the b-ARE motif as shown in Fig. 1A (nt 942-1050, GenBank accession number M14745), or 26-mer degenerated ORNs (degORNs) purchased from Dharmacon (Chicago, IL) were delivered to cells by *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (Roche Diagnostics, Indianapolis, IN). The transcription blocker 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was purchased from Sigma-Aldrich (St. Louis, MO).

Plasmids, Synthetic Transcripts, and Recombinant Proteins. The KpnI/HindIII 240-base pair fragment containing the SV40 promoter was excised from the pGL3-promoter vector and cloned into the corresponding restriction sites of the pGL4.71 vector (Promega, Madison, WI) to obtain the pGL4.71P plasmid. The 5′-primer CGTCTAGAGTCAACATGCCTGC and the 3′-primer CGTCTAGAGGTGATCCGGCCAA (flanked at the 5′-end by a CG overhang followed by the XbaI restriction site) were used to amplify the 400-base pair segment containing the 3′-UTR ARE sequence from the human bcl2 cDNA fragment. This fragment was cloned into the XbaI cloning site at the 3′-end of the hRlucP gene coding for *Renilla reniformis* luciferase of the pGL4.71P plasmid to produce the pGL4.71P b-ARE plasmid. Synthetic Bcl2 RNA (b-RNA) (540 nt) used for in vitro degradation assays was prepared as described previously (Bevilacqua et al., 2003a). For UV cross-linking assays, radiolabeled bcl2 transcript was synthesized as described previously (Chen et al., 2001). Proteins GST-KSRP, GST-HuR, and GST-AUF1(p37) were produced in BL21 and purified using glutathione affinity-Sepharose 4B (GE Healthcare, Little Chalfont, Buckinghamshire, UK) resin, whereas histidine-tagged TTP were produced in *Escherichia coli* and purified using glutathione affinity-Sepharose 4B or nickel-nitrilotriacetic acid (QIAGEN, Valencia, CA).

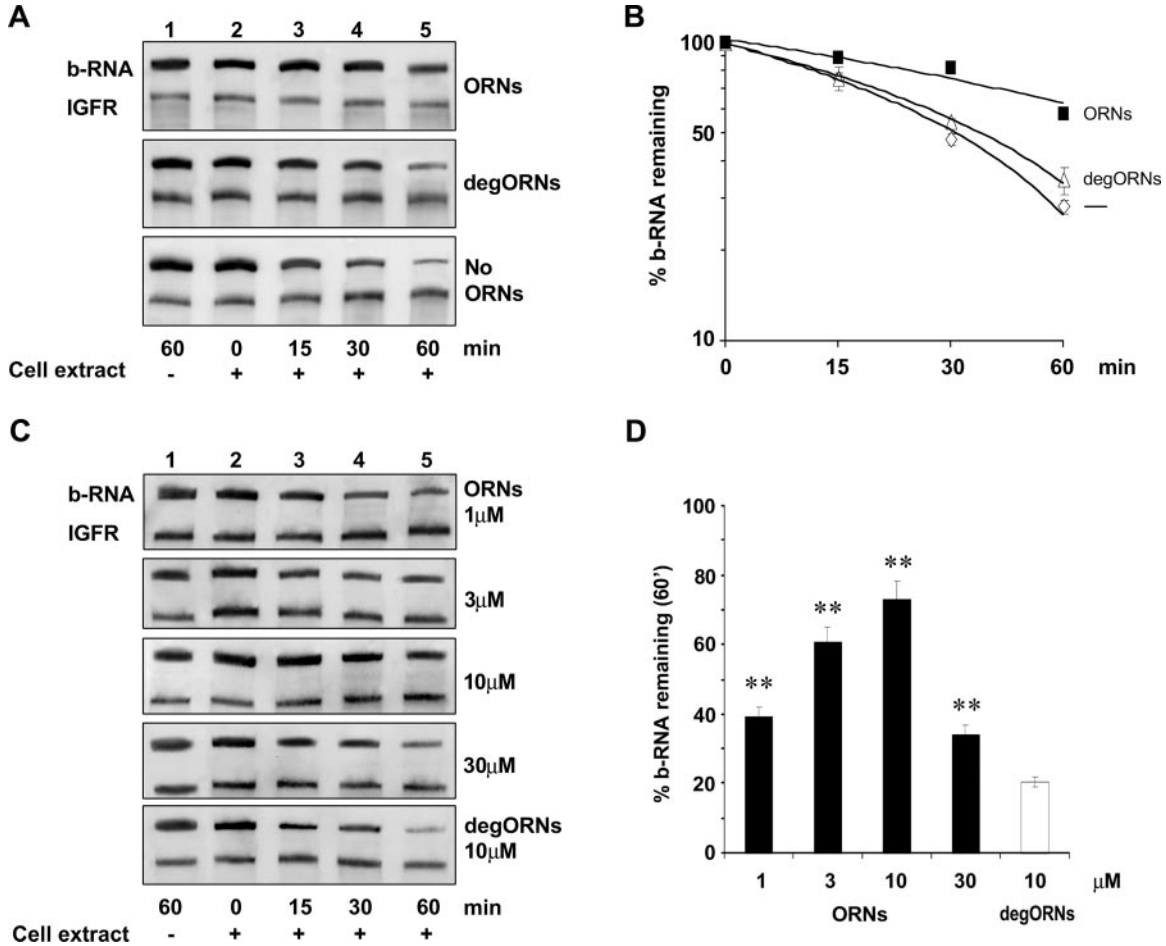

Fig. 2. Inhibition of b-RNA degradation by ORNs in vitro. A, the mixture of the three ORNs or the degORNs, total dose 10 μM, was added to the degradation mixture containing the total extract from the DOHH2 lymphoma cells, b-RNA (530 nt), and IGFR-RNA (350 nt). Incubation times with and without cell extract are indicated. B, time course of decay of b-RNA estimated by laser densitometry of the gel bands. Mean ± S.D. from three assays. C, dose-response assay of ORNs, experimental conditions as in A. D, histograms of C (incubation for 60 min at the indicated doses) obtained by densitometry (mean ± S.D., $n = 3$). Statistical significance was calculated versus degORN 10 μM treatment.

UV Cross-Linking. UV cross-linking assays were performed as described previously (Chen et al., 2001) with minor modifications. Each AUBP (KSRP, 400 ng; TTP, 300 ng; and HuR or p37-AUF1 200 ng) and ^{32}P-labeled b-RNA (0.5 ng = 2×10^5 cpm) were incubated at room temperature for 20 min in an RNA-binding buffer (20 μl) containing 10 mM HEPES, pH 7.6, 3 mM $MgCl_2$, 100 mM KCl, 2 mM dithiothreitol, 5% glycerol, 0.5% Nonidet P-40, 1 μg of yeast RNA, and 1 μg of heparin. For competition experiments, unlabeled ORNs (1, 10, and 100 μM) were incubated with lysates for 10 min before the addition of the RNA probe. Unbound RNA was digested with RNAase T1 (200 units per reaction) for 15 min at 37°C. Reaction mixtures were transferred to a 96-well plate and irradiated at 4°C for 10 min with a UV Stratalinker (Stratagene, La Jolla, CA). After digestion with RNase A (200 ng per reaction) for 10 min at 37°C, samples were separated by electrophoresis on reducing 10% SDS-polyacrylamide gels. Gels were dried, and ^{32}P-labeled proteins were made visible by autoradiography.

RNA Degradation. The assays were performed as described with minor modifications (Ghisolfi et al., 2005). The cell-free reaction mixture was maintained for 45 min at 4°C, before the activation at 37°C. Cellular degradation was studied in ORN-treated cells at day 0 and treated with DRB on day 1. After 0, 3, 5, and 7 h, total RNA was extracted with NucleoSpin RNA II columns (Macherey-Nagel, Easton, PA) and amplified by TaqMan real-time RT-PCR (Applied Biosystems, Foster City, CA). The cellular amounts of Bcl2, c-myc, mitogen-activated protein kinase kinase 6 (MKK6), and p27 mRNAs, normalized to the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene, were determined as above.

Cell Transfection. HEK 293 cells were cotransfected with pcDNA3 and pGL4.71P b-ARE plasmid expressing the chimeric mRNA Rluc-b-ARE by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 48 h, cells were replated in selective media containing 0.8 mg/ml antibiotic G418 (Sigma-Aldrich). Single clones were selected for their resistance to G418 and luciferase activity.

RNA Immunoprecipitation Assay. RNA immunoprecipitation was performed according to Gherzi et al. (2004) with slight modifications. In brief, HEK 293 cells, stably expressing pGL4.71P b-ARE plasmid, were transfected with ORNs at 1.5 μM. After 48 h, cells were lysed in a buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 100 mM NaF, 1 M $Na3VO_4$, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 10 mM vanadyl ribonucleoside complex, and 1 mg/ml aprotinin for 15 min at 4°C. Lysates, spun at 14,000*g* for 15 min at 4°C, and supernatants were incubated overnight with rabbit αKSRP (Gherzi et al., 2004) or TTP (purchased from Dr. W. Rigby, Dartmouth Medical School, Hanover, NH) or αHuR MoAb (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C under rotation and purified with protein A (rabbit antibodies) or Sepharose G (MoAb). RNA, extracted with RNAzol, was determined by real-time RT-PCR using an Rluc-b-ARE chimeric probe.

Western Blot Analysis. The assays were performed under standard conditions. Blots were probed with αBcl2 (Dako North America, Inc., Carpinteria, CA), αp27, and αHuR (Santa Cruz) MoAbs or rabbit αKSRP, αTTP, and $\alpha\beta$-actin (Sigma-Aldrich) and detected by ECL Plus kit (GE Healthcare).

Statistics. The data were expressed as means ± S.D., statistical significance was calculated by using a Student's *t* test, where $*$, $P \leq 0.05$ and $**$, $P \leq 0.01$.

Results

Displacement of ARE Binding Proteins. The turnover of many inducible RNAs is regulated by AUBPs. Three 26-mer ORNs, each homologous to one third of b-ARE core, shown in Fig. 1A, were used in UV cross-linking assays in an effort to obtain deeper insight into the mechanisms modulating bcl2 mRNA stability. Four relevant human recombinant AUBPs, KSRP, p37-AUF1, TTP, and HuR, were incubated with a radiolabeled fragment of bcl2 3′-UTR (b-RNA) encompassing the related b-ARE motif.

Oligoribonucleotides were added at 1 μM to the RNA-AUBP mixture in the reaction tube of the UV cross-linking assay. Figure 1B shows that each ORN inhibited the binding of AUBPs to the b-RNA in a sequence-specific fashion.

ORN2 was the most effective in displacing KSRP, HuR, and AUF1. In the TTP assay, the three ORNs inhibited the binding event with similar efficacy. Although UV cross-linking is only a semiquantitative assay, ORNs were able to displace AUF1, an effective regulator of the b-RNA half-life (Lapucci et al., 2002), in a dose-response fashion (Fig. 1C). To address the displacement activity in cells, HEK 293 cells stably transfected with pGL4.71P b-ARE plasmid expressing chimeric mRNA Rluc-b-ARE were treated with ORNs (1.5 μM). RNA immunoprecipitation assays were performed 30 h after transfection with ORNs to evaluate the effect on AUBPs binding to b-ARE. The amount of the relevant RNA was significantly reduced in cells treated with sense ORNs, as shown in Fig. 1D.

In Vitro Inhibition of b-RNA Degradation. Whether displacement of AUBPs might change the rate of b-RNA turnover was analyzed by a cell-free degradation assay. The b-RNA fragment including b-ARE, labeled by incorporation of the precursor DlG-UTP, was transcribed as reported previously (Bevilacqua et al., 2003a). An IGFR RNA fragment, labeled as above, was added to the degradation mixture as a control. For the decay assays, cell lysates from DOHH2 lymphoma cells were mixed with the relevant transcripts and incubated at 37°C for the indicated times.

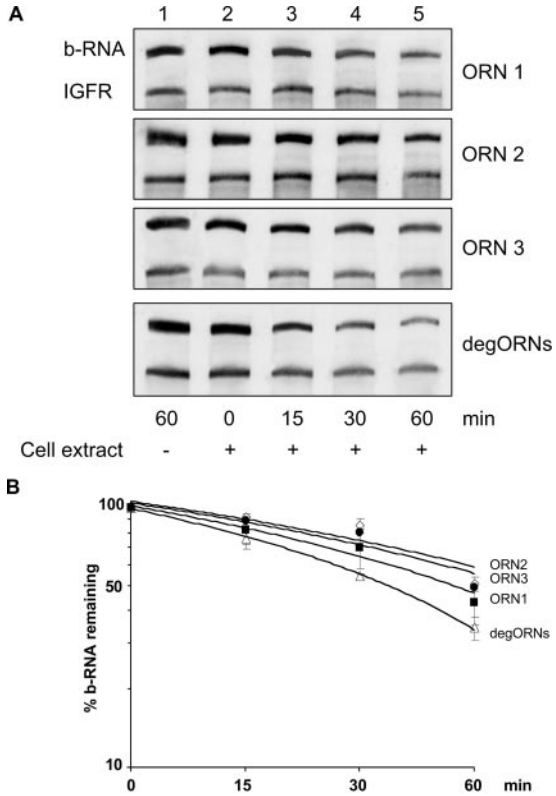

Fig. 3. Inhibition of b-RNA degradation by individual ORNs. A, as described in Fig. 2, with individual ORNs or degORNs at 10 μM. B, time course of decay of b-RNA estimated by laser densitometry of the gel bands (mean ± S.D., $n = 3$).

The three ORNs, used in combination to span the entire b-ARE motif at a final concentration of 10 μM, proved highly effective (Fig. 2A). Figure 2B shows that the half-life of the b-RNA was more than twice that of samples containing degORNs. In contrast, the degradation rate of the IGFR transcript, not expressing ARE motifs, was unaffected. The slowdown of b-RNA turnover by the ORNs was dose-dependent: the activity increased from 1 to 10 μM and decreased at higher doses (Fig. 2, C and D). Oligonucleotides designed in between ORN1 and ORN2 or ORN2 and ORN3 or flanking the core sequence did not display a significant activity (data not shown).

The displacing activity of each ORN at the optimal concentration of 10 μM was correlated with the rate of b-RNA degradation. As shown in Fig. 3A, at each time point, the rate of b-RNA degraded in the presence of each ORN at the optimal concentration of 10 μM. At each time point, the rate of b-RNA decay in the presence of ORNs was lower than with inactive degORNs. The most effective, ORN2, extended the half-life of b-RNA from 35 min to more than 60 min (Fig. 3B). ORN activity closely correlated with the displacing activity shown in the UV cross-linking assays.

Increased Level and Stabilization of RNAs in Cell Lines. Raji lymphoma cells and HL60 promyelocytic cells were exposed to three ORNs mixed to a total concentration of 1.5 μM in *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate. The amount of Bcl2 mRNA in the cells was measured by real-time RT-PCR (TaqMan Probe System) at the indicated times.

As shown in Fig. 4, A and C, ORNs were able to increase the amount of Bcl2 mRNA in both cell lines, with the highest activity being obtained 48 h after treatment. The possibility that ORNs might stabilize Bcl2 mRNA was studied by analyzing the amount of Bcl2 mRNA in extracts from cells treated with the transcription inhibitor DRB. Using the real-time RT-PCR, the Bcl2 mRNA half-life was calculated as 4.5 h in untreated cells and as more than 7 h in ORN-treated Raji cells (Fig. 4B); it was 3.5 h in untreated versus 6 h in

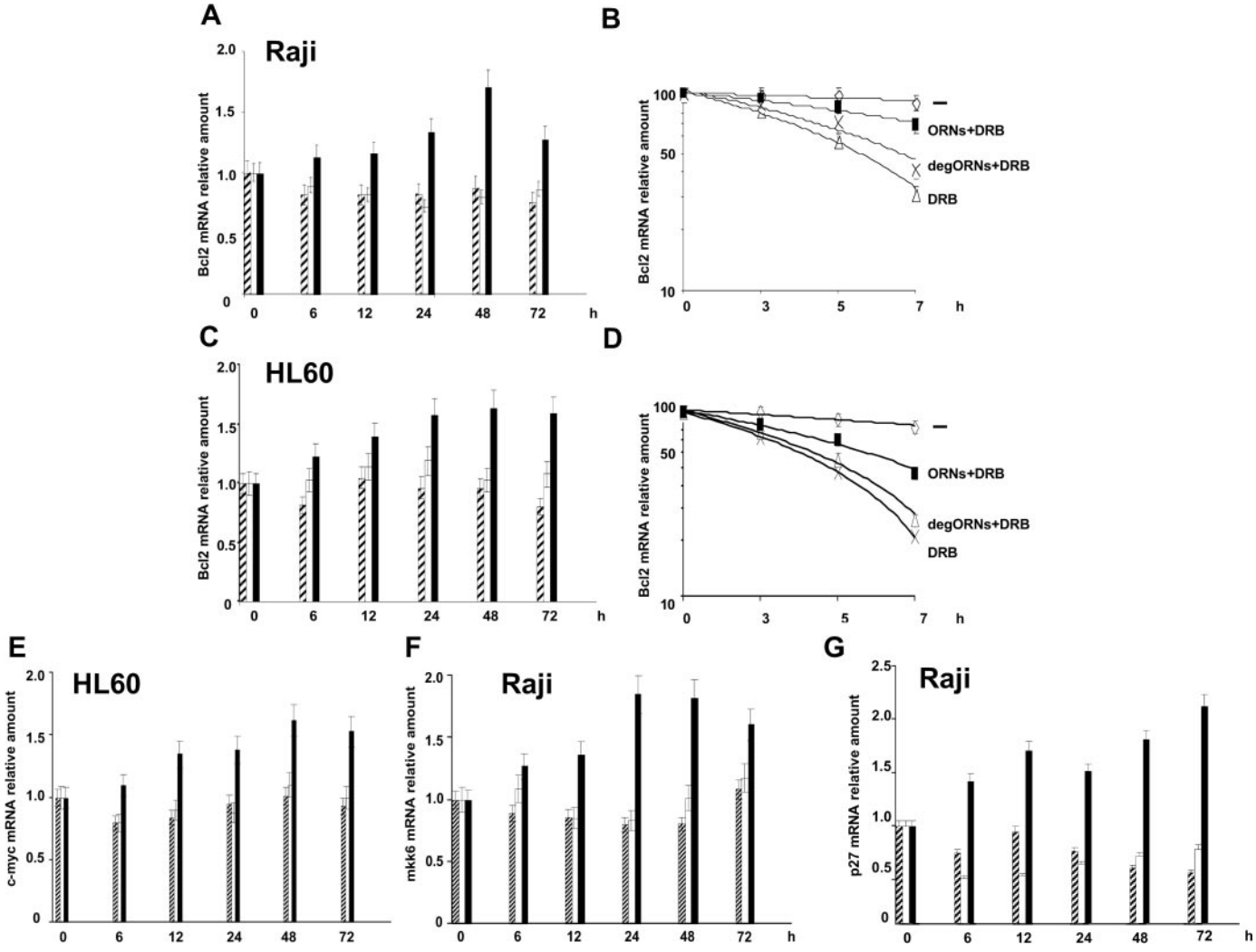

Fig. 4. ORN inhibition of Bcl2 RNA degradation in two cell lines. A, Bcl2 mRNA relative amount at different times in Raji cells exposed to ORNs. Cellular Bcl2 mRNA was determined by real-time RT-PCR in extracts from cells transfected with 1.5 μM ORNs at time 0, normalized with glyceraldehyde-3-phosphate dehydrogenase cDNA (mean ± S.D., $n = 3$). B, rate of bcl2 mRNA degradation in Raji cells after treatment with the transcription inhibitor DRB (20 μg/ml). C, relative amount of bcl2 mRNA at different times in HL60 cells since the ORN treatment. D, rate of Bcl2 mRNA degradation in HL-60 cells after treatment with the transcription inhibitor DRB (20 μg/ml). E, amount of c-myc mRNA in HL60 cells at different times since the ORN treatment. F, time course of MKK6 mRNA in Raji cells. G, time course of p27 mRNA in Raji cells. A, C, E, F, and G: ▨, untreated; □, degORN; ■, ORNs. For all panels, data are expressed as mean ± S.D., $n = 3$.

ORN-treated HL60 cells (Fig. 4D). Individual AUBPs can interact and regulate the mRNA decay of many genes (Chen and Shyu, 1995); it is thus possible that the AUBPs displacement can stabilize ARE-expressing genes other than bcl2 (Supplemental Data S1). Therefore, we measured the amount of c-myc RNA in HL60 cells (Fig. 4E) and the amount of MKK6 and p27 RNA in Raji cells (Fig. 4, F and G). The amount of all three RNAs was significantly increased in cells treated with sense-oriented ORNs.

Level of Relevant Proteins in Cells Treated with ORNs. The increased level of RNAs in ORN-treated cells prompted us to study the expression of the relevant proteins in two lymphoid cell lines. Figure 5A shows the increased expression of Bcl2 protein measured on day 3 or 5 from the initial exposure to the ORNs. Proteins were quantified by densitometry and plotted in histograms (Fig. 5B). The dose-related activity peaked at 1 μM, was lower at 3 μM, and was higher on day 5 (after two ORN treatments). In a similar way, the level of p27 protein, whose RNA expresses an ARE motif, was significantly increased by the ORN treatments (Fig. 6, A and B).

Discussion

Synthetic single-strand oligoribonucleotides, chemically modified to resist nuclease degradation, were prepared to compete with b-ARE for some relevant AUBPs, in accordance with the decoy-aptamer strategy (Tomita et al., 2002; Nimjee et al., 2005). We also examined whether sense-oriented ORNs would change the rate of b-RNA degradation in in vitro assays and in viable cells.

In direct UV cross-linking assays, ORNs successfully competed with the b-ARE motif, probably inhibiting the interaction of recombinant HuR, KSRP, AUF1, or TTP with b-RNA. Although both stabilizing and destabilizing AUBPs were displaced by oligonucleotides, RNA stabilization turned out as the final effect of ORN treatment.

ORN2, designed on the central region of the ARE motif, was the most effective to inhibit the binding of all four AUBPs. In this subregion are located relevant determinants for the degradation machinery, determinants that are required for the binding of important AUBPs. The binding activity of HuR and KSRP to the b-ARE was strongly decreased also in HEK 293 cells upon ORNs treatment, as shown by RNA immunoprecipitation.

Each AUBP, including those studied here, can bind AREs present in several mRNAs (Good, 1995; Lu and Schneider, 2004). How one AUBP can bind different nucleotide structures is not yet known. A possibility is the recognition of characterizing elements such as the AUUUA pentamer or U-rich stretches expressed in the 3′-UTR of many messengers (Winzen et al., 2004; Fialcowitz et al., 2005). According to the post-transcriptional operon model (Keene and Lager, 2005), RNA binding proteins coordinately regulate the expression of multiple mRNAs encoding functionally related proteins. These mechanisms provide combinatorial regulation of genetic information, including the evolution of multifunctionality of eukaryotic proteins. Systems and high-throughput approaches have provided insight into coordinated post-transcription regulation of gene expression. Multiple interactions have been shown by micro-RNAs, which can also complement short 3′-UTR stretches (5–8 nt) within a variety of RNA targets contributing to regulate protein synthesis along a coordinated fashion (Yoon and De Micheli, 2005; Kim and Nam, 2006). This study, although not involved directly into this matter, suggests that characterizing structures expressed in most AREs may behave as shared targets for AUBPs.

The binding of a protein to different AREs of unrelated transcripts was inhibited by sequence-specific ORNs mimicking endogenous structures. Meanwhile, one single ORN can compete with different AUBPs.

The decoy activity in vitro and in cell lines actually turned

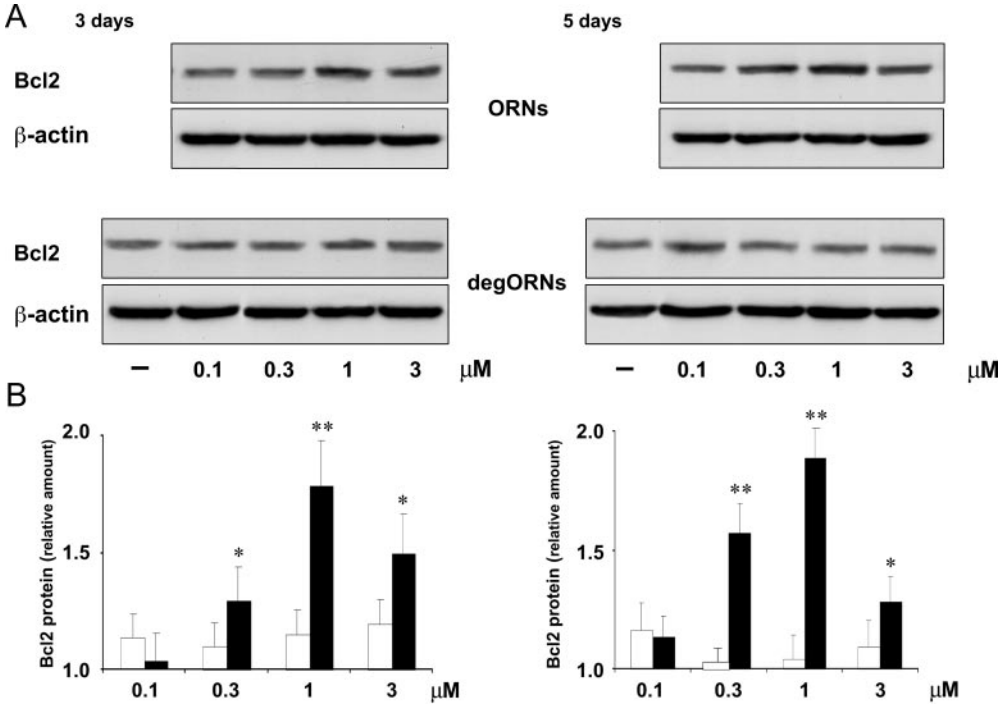

Fig. 5. Bcl2 protein content in HL60 cell treated with ORNs. A, ORNs or degORNs were transfected into HL60 cells, at the indicated dose, at day 0 and at half-dose at day 3 in the 5-day assays, under the same conditions as described in Fig. 4. Cell samples were analyzed for Bcl2 protein levels by Western blot assays on days 3 and 5. B, the histograms show the relative intensity versus untreated (□, degORNs; ■, ORNs) of the gel bands in A, estimated by laser densitometry (mean ± S.D., $n = 4$).

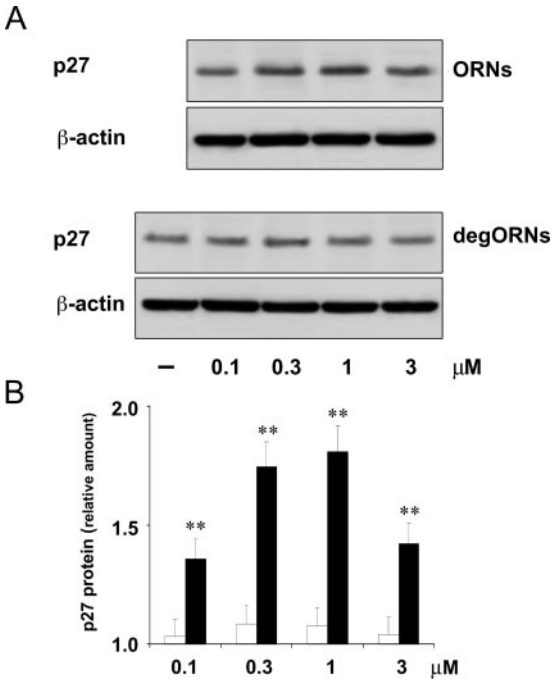

Fig. 6. Amount of p27 protein in Raji cells treated with ORNs. A, Western blot analysis of p27 protein amount in Raji cells; experimental conditions as described in Fig. 5, assays on day 3. B, the histograms show the relative intensity versus untreated (□, degORNs; ■, ORNs) of the gel bands in A, estimated by laser densitometry (mean ± S.D., $n = 3$).

out to regulate the rate of b-RNA degradation. In the cell-free system, ORNs protected b-RNA from the degradative action of an effective cell extract. The most effective individual ORN was ORN2, although ORN3 showed no detectable activity. Thus, the stabilizing activity mirrored the decoy activity. Moreover, the effect of the triple ORN mixture on the rate of b-RNA degradation was dose-dependent. This suggests that the decreased interaction of destabilizing AUBPs with Bcl2 mRNA transcript, due to the ORN presence, leads to an mRNA stabilization.

In different human cell lines, ORNs were able to significantly increase the level of Bcl2 mRNA. The biochemical mechanism might be dependent on the ORN ability to slow down the rate of degradation, as shown by the time course determinations of the Bcl2 mRNA in cells treated with a transcription inhibitor.

Not entirely unexpected, ORNs also inhibited the rate of degradation of unrelated mRNAs whose decay is dependent on AREs and AUBPs. These observations might figure out the coordinated and temporally linked regulation of functionally related transcripts.

Consistent with the increased amount of mRNA, the level of Bcl2 protein was significantly and dose-dependently higher in cells exposed to ORNs. Moreover, as predicted above, the level of proteins encoded by stabilized RNAs were increased as well. It is shown that the p27 protein, which fluctuates during the cell cycle often in coordination with the level of Bcl2 (Calastretti et al., 2001; Greider et al., 2002), was also up-regulated. Although the mechanism of RNA augmentation by ORNs homologous to the b-ARE motif is only in part understood, the findings described here are in keeping with a model in which ARE is regulated at different levels of specificity and coordination (Keene and Lager, 2005).

The ARE of the bcl2 gene, characterized by a unique nucleotide sequence, might be targeted specifically by the relevant protein that actually regulates the rate of degradation of its own RNA, according to a negative feedback mechanism (Bevilacqua et al., 2003a). The bcl2 transcript can also be regulated specifically by antisense-oriented oligoribonucleotides targeting the b-ARE motif (Ghisolfi et al., 2005), supporting the concept of the individual regulation.

Conversely, the coordinated regulation of multiple related transcripts, as shown here by the use of sense-oriented ORNs, is not in contrast with the single transcript regulation. On the basis of studies on AUBP displacement, sequence-specific ORNs, by competing for the docking sites of proteins that recognize common elements within the ARE structures, might exert a multitranscript activity. These findings support a combinatorial modality for the coordinating transcript regulation based on the interaction of *trans*-acting factors and *cis*-elements.

Acknowledgments

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