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AUF1 Is a *bcl-2* A + U-rich Element-binding Protein Involved in *bcl-2* mRNA Destabilization during Apoptosis*

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We previously identified a conserved A + U-rich element (ARE) in the 3′-untranslated region of *bcl-2* mRNA. We have also recently demonstrated that the *bcl-2* ARE interacts with a number of ARE-binding proteins (AUBPs) whose pattern changes during apoptosis in association with *bcl-2* mRNA half-life reduction. Here we show that the AUBP AUF1 binds *in vitro* to *bcl-2* mRNA. The results obtained in a yeast RNA three-hybrid system have demonstrated that the 1–257-amino acid portion of p37 AUF1 (conserved in all isoforms), containing the two RNA recognition motifs, also binds to the *bcl-2* ARE *in vivo*. UVC irradiation-induced apoptosis results in an increase of AUF1. Inhibition of apoptosis by a general caspase inhibitor reduces this increase by 2–3-fold. These results indicate involvement of AUF1 in the ARE/AUBP-mediated modulation of *bcl-2* mRNA decay during apoptosis.

The *bcl-2* gene encodes the multifunctional Bcl-2 protein known to be involved in cell growth, differentiation control, and prevention of apoptosis (1). Down-regulation of *bcl-2* expression is a general response of the cell to apoptotic stimuli (2, 3). The mechanisms by which Bcl-2 exerts a protective activity against apoptosis are still unclear, but the mechanism by which *bcl-2* expression is regulated has recently been partially elucidated. A large amount of evidence indicates that up- and down-regulation of *bcl-2* expression is modulated both at transcriptional and posttranscriptional levels, the latter of which includes mRNA stability and protein activity control. Expression of the *bcl-2* gene was known to be regulated transcriptionally by a negative regulatory element (4). Two estrogen-responsive elements within the coding region involved in transcriptional regulation of *bcl-2* have also been characterized recently in a breast cancer cell line (5). One of the posttranscriptional control mechanisms of *bcl-2* expression has been described to be mediated by phosphorylation of Bcl-2 protein at different amino acid positions (6, 7). Another posttranscriptional mechanism of *bcl-2* regulation has been identified in our laboratory. This mechanism modulates *bcl-2* mRNA stability and involves a cis-acting A + U-rich element (ARE)[1] located in the 3′-UTR of *bcl-2* mRNA (8) that binds to a number of ARE-binding proteins (AUBPs) whose pattern undergoes modifications during apoptosis in association with *bcl-2* mRNA decay (9).

AREs represent a class of cis-acting elements that modulate mRNA stability (10). They are present in a variety of mRNAs of genes required to be rapidly and finely modulated under particular conditions, such as response to growth factors, serum starvation, and apoptosis (8, 11–13). *GM-CSF* and c-*fos* mRNAs were the first to be studied in detail (14, 15). Compared with the AREs of these genes (16), the *bcl-2* ARE possesses a moderate, constitutive, destabilizing activity that is dramatically enhanced upon application of apoptotic stimuli (8, 9).

The mechanism of action of ARE-mediated mRNA decay is under investigation by many laboratories. A number of AUBPs acting as trans-acting factors are known. In particular, some AUBPs belonging to the heterogeneous nuclear ribonucleoprotein family are involved in mRNA localization and stability control (17, 18). Among them, AUF1 and the embryonic lethal abnormal vision-like protein HuR are able to enhance or inhibit mRNA degradation, respectively. For example, there is evidence for binding and stabilization of c-*fos*, plasminogen activator inhibitor 2, and vascular endothelial growth factor mRNAs by HuR (19–23). This protein has also been implicated in the increase of p53-induced $p21^{waf1}$ mRNA after apoptotic stimuli (24). AUF1 was first identified as an mRNA-binding protein with selective affinity for AREs located within mRNAs such as c-*myc*, c-*fos,* and *GM-CSF* (25, 26). Although its destabilizing function is well documented, some studies suggest that AUF1 may have a role in the stabilizing complex on the α-globin mRNA (27) and, more recently, as a parathyroid hormone mRNA-binding protein that modulates mRNA stability (28). AUF1 is comprised of four isoforms of 37, 40, 42, and 45 kDa (25, 29, 30). Although the role of each isoform has yet to be fully characterized, a direct correlation has been observed between each AUF1 isoform's binding affinity and its RNA-destabilizing

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[1] The abbreviations used are: ARE, A + U-rich element; AUBP, ARE-binding protein; THS, three-hybrid system; REMSA, RNA electrophoretic mobility shift assay; Ab, antibody; UTR, untranslated region; TK, transketolase; nt, nucleotide(s); Z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone.

FIG. 1. **Schematic diagram of *bcl-2* mRNA and sequences of ARE segments used for either *in vitro* transcription or cell transfection.** *bcl-2 ARE+,* a 396-nt segment of *bcl-2* 3′-UTR ranging from nucleotide 752–1147 of the *bcl-2* cDNA sequence (GenBank™ accession number M14745) and containing the ARE. *bcl-2 ARE,* an 107-nt ARE that is a segment of *bcl-2* 3′-UTR ranging from nucleotide 944–1050 of the *bcl-2* evolutionary conserved cDNA sequence and harboring A + U-rich motifs. *bcl-2 ΔARE,* a 289-nt segment of *bcl-2* 3′-UTR ranging from nucleotide 752–1147 of the *bcl-2* cDNA sequence (GenBank™ accession number M14745) carrying a deletion of 107 bp from nucleotide 944–1050 corresponding to the ARE core.

activity toward different AREs, with isoforms p37 and p42 being the most effective (31).

Here, we demonstrate that AUF1 is a *bcl-2* mRNA-binding protein and that potentially all its isoforms are able to form complexes with the *bcl-2* ARE. At doses able to induce apoptosis, UVC irradiation induced an increase of cytoplasmic levels of the p45 AUF1 isoform, which was paralleled by enhancement of a *bcl-2* mRNA-AUF1 complex and mediated by a mechanism that requires caspase activation. These results indicate that ARE-mediated *bcl-2* mRNA down-regulation during apoptosis involves AUF1 and suggest different roles for its four isoforms.

EXPERIMENTAL PROCEDURES

Plasmids Used for in Vitro Transcription and Transfection—The schematic diagram of *bcl-2* mRNA and the sequences of ARE segments used for either *in vitro* transcription or cell transfection are shown in Fig. 1. A 396-bp segment encoding the human *bcl-2* mRNA located in the 3′-UTR from nucleotide 752–1147, named *bcl-2* ARE+ (GenBank™ accession number M14745; Ref. 32), was obtained by PCR amplification using the plasmid pBS-SK-H-Bcl-2 (33) as template, with 5′- AGTCAACATGCCTGC-3′ forward (FW1) and 5′- GTGATCCGGCCAACAAC-3′ reverse (RV2) primers. The *bcl-2* ARE+ was cloned in the TA cloning site of the pCRII plasmid according to the TA Cloning Kit specifications (Invitrogen), yielding pCRII/*bcl-2* ARE+, and used to synthesize the *bcl-2* ARE+ riboprobe. A 289-bp segment of the human *bcl-2* mRNA located in the 3′-UTR from nucleotide 752–1147, named *bcl-2* ΔARE, was obtained by nested deletion of the cDNA from nucleotide 944–1050 (32) using the plasmid pBS-SK-H-Bcl-2 (33) as template. Two partially overlapping PCR products were synthesized for this purpose. The first PCR product was amplified with FW1, as described above, and 5′-TTCGACGTTTTGCCTGAAGACT-3′ reverse (RV1) primers. The second PCR product was amplified with 5′- CAAAACGTCGAACGACCACTAATTGCCAAGC -3′ (FW2) and RV2 primers. The 12 overlapping nucleotides are *underlined* in RV1 and FW2 primers. The segment *bcl-2* ΔARE was cloned in plasmid pCRII as described above, yielding pCRII/*bcl-2* ΔARE, and used for the synthesis of *bcl-2* ΔARE riboprobe. A 107-bp segment of human *bcl-2* mRNA located in the 3′-UTR from nucleotide 944–1050 (a short region with the highest evolutionary conservation containing AUUUA pentamers and UUAUUUAUU nonamer particularly rich in A + U motifs and therefore considered the ARE core), named *bcl-2* ARE (32), was obtained by PCR amplification using the plasmid pBS-SK-H-Bcl-2 (33) as template, with 5′-TCAGCTATTTACTGCCAAAG-3′ forward and 5′-GATTTCCAAAGACAGGAG-3′ reverse primers. The *bcl-2* ARE product was cloned in pCRII as described above, yielding pCRII/*bcl-2* ARE to synthesize the *bcl-2* ARE riboprobe. The remaining polylinkers of pCRII/*bcl-2* ARE+, pCRII/*bcl-2* ΔARE, and pCRII/*bcl-2* ARE were removed by *Apa*I digestion and religation. Plasmids pBBB4 (obtained by Dr. Ann-Bin Shyu, University of Texas, Houston Health Science Center, Houston, TX) and pBBB-U1 have been described previously (8). Plasmid pBBB-U2 was obtained by cloning the *Bgl*II/*Bam*HI segment of pCRII/*bcl-2* ΔARE into the unique *Bgl*II site of plasmid pBBB4. Plasmids pBBB4, pBBB-U1, and pBBB-U2, used for RNase protection analysis of mRNA stability, contain the rabbit β-globin gene transcriptionally driven by the serum-inducible c-*fos* promoter (pBBB4) with inserted the *bcl-2* ARE (pBBB-U1) or *bcl-2* ΔARE (pBBB-U2).

Cell Lines and Transfections—The Jurkat T-cell leukemia line (clone E61; European Collection of Animal Cell Cultures) was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin in a humid-

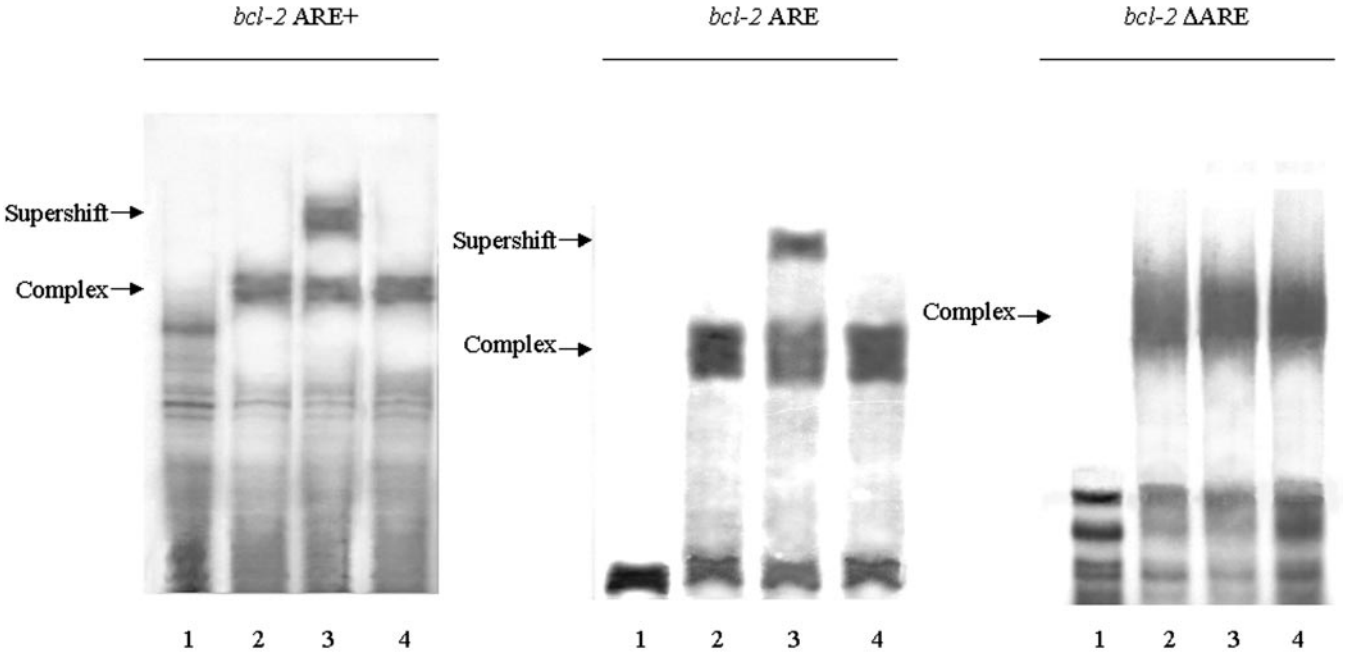
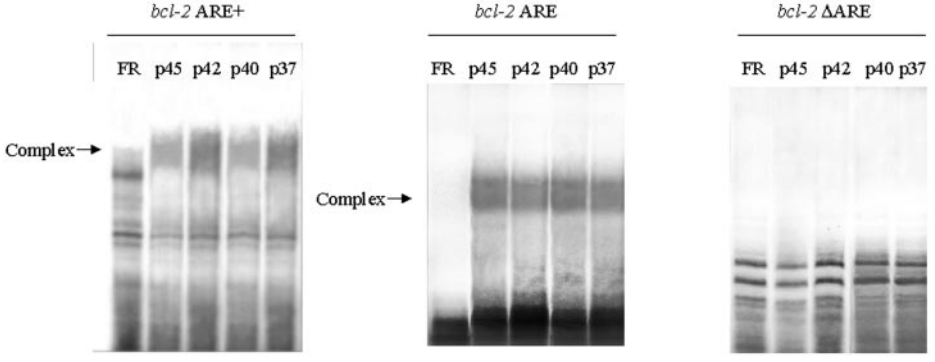


FIG. 2. **Detection of AUF1 binding to the *bcl-2* ARE by REMSA/supershift.** *Left panel*, REMSA/supershifts with *bcl-2* ARE+. *Lane 1*, *bcl-2* ARE+ riboprobe; *lane 2*, *bcl-2* ARE+ riboprobe incubated with cytoplasmic extracts; *lane 3*, *bcl-2* ARE+ riboprobe incubated with cytoplasmic extracts and anti-AUF1 Ab; *lane 4*, *bcl-2* ARE+ riboprobe incubated with cytoplasmic extracts and anti-TK Ab. *Center panel*, REMSA/supershifts with *bcl-2* ARE. *Lane 1*, *bcl-2* ARE riboprobe; *lane 2*, *bcl-2* ARE riboprobe incubated with cytoplasmic extracts; *lane 3*, *bcl-2* ARE riboprobe incubated with cytoplasmic extracts and anti-AUF1 Ab; *lane 4*, *bcl-2* ARE riboprobe incubated with cytoplasmic extracts and anti-TK Ab. *Right panel*, REMSA/supershifts with *bcl-2* ΔARE. *Lane 1*, *bcl-2* ΔARE riboprobe; *lane 2*, *bcl-2* ΔARE riboprobe incubated with cytoplasmic extracts; *lane 3*, *bcl-2* ΔARE riboprobe incubated with cytoplasmic extracts and anti-AUF1 Ab; *lane 4*, *bcl-2* ΔARE riboprobe incubated with cytoplasmic extracts and anti-TK Ab. *Lane 1* indicates free RNA digested with RNase T1. Complexes and supershifts are indicated.

FIG. 3. **Binding of all AUF1 isoforms to the *bcl-2* ARE.** All AUF1 isoforms synthesized *in vitro* were assayed in REMSA experiments for RNA binding activity with the three ARE riboprobes. *Left panel,* the *bcl-2* ARE+ riboprobe incubated with each AUF1 isoform. *Center panel,* the *bcl-2* ΔARE riboprobe incubated with each AUF1 isoform. *Right panel,* the *bcl-2* ARE riboprobe incubated with each AUF1 isoform. *FR* represents free RNA digested with RNase T1. *p37, p40, p42,* and *p45* indicate each AUF1 isoform used in shift experiments. Complexes are indicated.



ified atmosphere, 5% CO_2, at 37 °C. The two mouse fibroblast NIH 3T3 polyclonal cell lines transfected with plasmids pBBB4 or pBBB-U1 have been described previously (8). The third NIH 3T3 polyclonal cell line was obtained upon transfection with plasmid pBBB-U2 described above. All NIH 3T3 cell lines were maintained in Dulbecco's modified Eagle's medium supplemented as described above for RPMI 1640 medium.

RNase Protection Assays—Reporter β-globin mRNA stability was determined by the serum-inducible transcriptional pulse system reported previously (8).

In Vitro Transcription—The plasmids pCRII/*bcl-2* ARE+, pCRII/*bcl-2* ΔARE, and pCRII/*bcl-2* ARE were linearized with *Sma*I and used for *in vitro* run-off transcription from the T7 promoter using an RNA labeling Kit (Amersham Biosciences) in the presence of [α-^{32}P]UTP (800 Ci/mmol; Amersham Biosciences) to obtain three radiolabeled *bcl-2* ARE riboprobes (Fig. 1).

In Vitro Translation—The isoforms of AUF1 were prepared from 1 μg of each of four different pcDNA-AUF1 plasmids (p45, p42, p40, and p37). Templates p45 and p42 were prepared by *Not*I digestion, whereas templates of p37 and p40 were prepared by *Apa*I digestion. The cDNAs were *in vitro*-transcribed/translated in the presence or absence of [^{35}S]methionine (Amersham Biosciences) with the TnT-coupled reticulocyte lysate system (Promega, Madison, WI) according to the manufacturer's instructions.

RNA Electrophoretic Mobility Shift Assay (REMSA) and REMSA/Supershift—Cells (10^7) induced to apoptosis by irradiation with UVC (15 J/m^2, 254 nm), with or without a 2-h pretreatment with 100 μM Z-VAD-fmk (Bachem AG), were collected at various time points, washed with ice-cold phosphate-buffered saline, and lysed in 100 μl of lysis buffer (10 mM HEPES, pH 7.9, 40 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol, 0.2% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) for 10 min on ice. Nuclei were pelleted by centrifugation at 14,000 rpm for 30 s in a microcentrifuge, and extracts were processed immediately or stored at −70 °C. REMSAs were performed by incubating the radiolabeled *bcl-2* ARE+, *bcl-2* ΔARE, and *bcl-2* ARE riboprobes (5×10^5 cpm) with cytoplasmic protein extracts (30 μg, determined by BCA reagent; Pierce) or with *in vitro* synthesized AUF1 isoforms in a reaction mixture (20 μl) containing 10 mM Tris, pH 7.5, 0.1 M potassium acetate, 5 mM magnesium acetate, 2 mM dithiothreitol, 15 units of RNasin (Promega), and 50 μg of heparin for 20 min at room temperature, followed by digestion for 20 min at room temperature with 5 units of RNase T1 (La Roche Ltd.), which cuts RNA downstream to guanosine residues in a number of fragments. Samples were separated on a native polyacrylamide gel (6% polyacrylamide:bisacrylamide, 60:1). In REMSA/supershift experiments, RNase T1 was added after incubation of samples with 50 μg/ml polyclonal rabbit anti-AUF1 antibody (Ab) or with nonspecific control anti-transketolase (TK) polyclonal Ab, a kind gift of

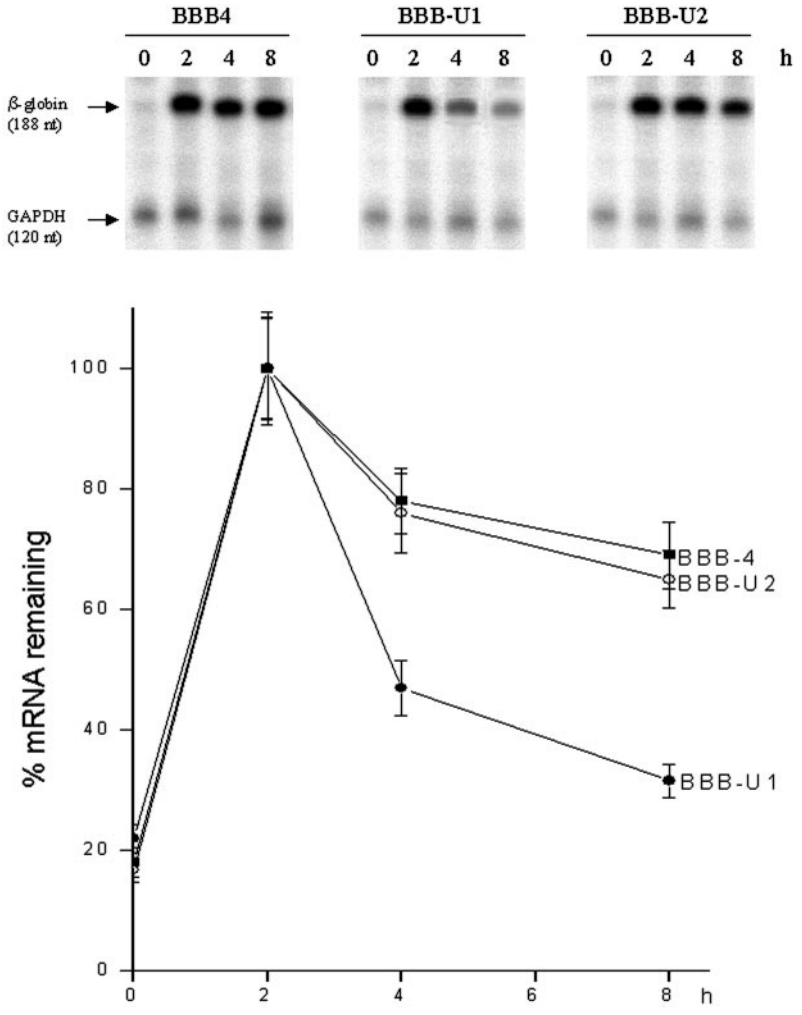


FIG. 4. **Decay of β-globin mRNAs containing *bcl-2* 3′-UTR sequences.** NIH 3T3 cells were stably transfected with the chimeric constructs pBBB4 (rabbit β-globin gene), pBBB-U1 (*bcl-2* ARE+ in rabbit β-globin 3′-UTR), and pBBB-U2 (*bcl-2* ΔARE in rabbit β-globin 3′-UTR). After serum starvation, 15% fetal calf serum was added to induce a pulse of transcription, and total RNA was extracted at the indicated time points. Autoradiograms show the representative PAGE analysis after RNase protection assays using two probes containing a 188-nt segment of the rabbit β-globin mRNA or a 120-nt segment of the murine glyceraldehyde-3-phosphate dehydrogenase mRNA, respectively. Phosphorimager-derived values of the rabbit β-globin protected segments have been normalized for the relevant values of the endogenous glyceraldehyde-3-phosphate dehydrogenase protected segment. Results shown on the graph are the means ± S.E. of three independent experiments.

Prof. F. Paoletti (University of Florence), for 20 min at room temperature, electrophoresed as described above, and exposed to Hyperfilm MP (Amersham Biosciences).

Western Blot Analysis—Cytoplasmic proteins (50 μg, prepared as described above) were separated by 12.5% SDS-PAGE, electroblotted (Miniprotean apparatus; Bio-Rad) onto Hybond-C membrane (Amersham Biosciences), and detected with ECL Western blotting analysis system (Amersham Biosciences) with 300 ng/ml anti-AUF1 Ab. Each isoform was identified on the basis of molecular mass markers (Bio-Rad).

UV-Cross-linking Assay and Immunoprecipitation—Aliquots of cytoplasmic proteins (50 μg, prepared as described above) were incubated with the ^{32}P-labeled *bcl-2* ARE riboprobe (10^9 cpm/μg, 5×10^5 cpm) in the presence of 0.5 mg/ml heparan sulfate and 2 μg of tRNA in a microplate (total volume, 10 μl) at room temperature for 10 min. RNA-protein complexes were cross-linked on ice by exposure to UVC for 5 min with 3000 μW/cm^2 in a Stratalinker 1800 (Stratagene). Samples were incubated with RNase A (1 μl, 1 mg/ml) for 30 min at 37 °C to digest unbound RNA. Proteins were immunoprecipitated by a 1-h incubation with polyclonal anti-AUF1 rabbit Ab (10 μg/ml), followed by overnight incubation with protein A-agarose (Sigma) at 4 °C. The immunoprecipitates were separated by 12.5% SDS-PAGE, autoradiographed, and analyzed by a Storm PhosphorImager (Molecular Dynamics). The molecular mass of each complex was evaluated on the basis of the *in vitro*-synthesized radiolabeled isoforms used as external standards and molecular mass markers (Bio-Rad).

The RNA Three-hybrid System (THS)—The yeast strain L40-coat and plasmids pIIIA/MS2-1, pIIIA/IRE-MS2, pAD-IRP1, and pACT2 (34) were gifts from Dr. M. Wickens (University of Wisconsin). The plasmid pRevR2 (35) was a gift from Dr. U. Putz (University of Hamburg, Hamburg, Germany). The hybrid RNA vector pIIIA/MS2-B2ARE harbors the 107-nucleotide sequence of the *bcl-2* ARE region. The sequence was PCR-amplified from pBS-SK-H-Bcl-2, with 5′-GACCCGGGTCAGCTATTTACTGCCAAAG-3′ forward and 5′-GACCCGGGGATTTCCAAAGACAGGAG-3′ reverse primers, subcloned in pCRII vector (Invitrogen), and inserted into the *Sma*I site of plasmid pIIIA/MS2-1. The resulting plasmid, pIIIA/MS2-bcl2, was transformed into L40-coat, and the chimeric RNA levels were assayed by Northern analysis (data not shown). To prevent transcription termination due to an RNA polymerase III termination signal in the poly-U stretch, a mutation at nucleotide 981 was introduced by PCR. The mispaired primers 5′-CATTTATTTgTTACATTATTAAG-3′ (forward) and 5′-CTTAATAATGTAAcAAATAAATG (reverse) were used to insert a single-base substitution (T→G, as indicated by *lowercase letters*), and the resulting segment was cloned into the pIIIA/MS2-1 plasmid as described previously. The AUF1 cDNA corresponding to the first 257 amino acids of p37 was amplified from pBAD/HISB-p37^{AUF1} (36) with the 5′-GAGGATCCGAATGTCGGAGGAGCAG-3′ forward and 5′-GACTCGAGTCTTCCTGCAAATCCTCC-3′ reverse primers to test the interaction between AUF1 and *bcl-2* ARE. The PCR product was digested and inserted into the *Bam*HI/*Xho*I sites of pACT2, in-frame with the GAL4 activation domain.

RESULTS

AUF1 Is a bcl-2 ARE-binding Protein Related to bcl-2 mRNA Half-life Regulation—In previous work (9), we demonstrated that the mRNA-destabilizing ARE of *bcl-2* binds specifically to cytoplasmic AUBPs, whose pattern undergoes modifications during apoptosis. The observation that proteins ranging from 30–50 kDa underwent the most noticeable increase led us to hypothesize that AUF1 could be a *bcl-2* ARE-binding protein. To examine this possibility, REMSA supershift analysis of cytoplasmic protein complexes with *bcl-2* ARE radiolabeled riboprobes (Fig. 1) has been carried out using an anti-AUF1 Ab and a nonspecific control anti-TK Ab (Fig. 2). When *bcl-2* ARE+ or *bcl-2* ARE riboprobes are used, the anti-AUF1 Ab (*lane 3* of each panel), but not the nonspecific anti-TK Ab (*lane 4* of each

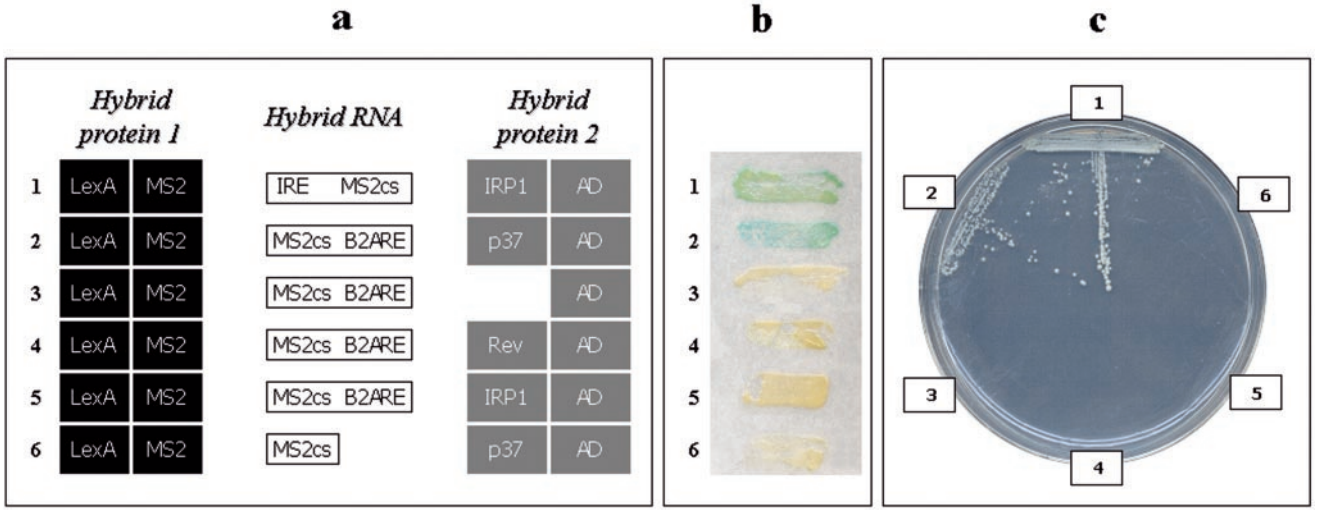


FIG. 5. **Detection *in vivo* of p37 AUF1-ARE interaction by RNA THS.** The RNA THS was used to study the interaction of AUF1 with the *bcl-2* ARE *in vivo*. We tested the indirect interaction between GAL4 activating domain (*AD*) fused to 1–257 amino acids of p37 AUF1 (*p37*) and LexA binding domain (*LexA*) fused to MS2 coat protein (*MS2*) by means of a bridging chimeric RNA containing the *bcl-2* ARE (*B2ARE*) and MS2 consensus sequence (*MS2cs*). The interaction is documented by transcription of the reporter genes LacZ and HIS3. The two components present in each chimeric factor of the system are depicted in the proper polarity, either N-terminal to C-terminal or 5′ to 3′. Fusion proteins are depicted in *boxes* (*black* and *gray boxes* for hybrid protein 1 and hybrid protein 2, respectively), with each box corresponding to a single domain. p37 AUF1 (*a*, *row 2*) is able to activate LacZ gene (*light blue-stained colonies*; *b*, *row 2*) or HIS3 gene transcription (growth on −Leu, −His medium containing 3 mM 3-aminotriazole; *c*, *sector 2*) when the B2ARE-containing transcript is expressed, but not in the presence of transcript lacking the B2ARE (*a* and *b*, *row 6*; *c*, *sector 6*). Iron response protein 1 (*IRP1*) is a positive control when assayed for binding to iron-responsive element (*IRE*) (*a* and *b*, *row 1*; *panel c*, *sector 1*) or a negative one when assayed for binding to B2ARE (*a* and *b*, *row 5*; *c*, *sector 5*). HIV-1 Rev is a RNA regulatory element-interacting protein used as a negative control when assayed for binding to B2ARE (*a* and *b*, *row 4*; *panel c*, *sector 4*). AD alone provided an additional negative control (*a* and *b*, *row 3*; *panel c*, *sector 3*). Furthermore, the utilized yeast strain (L40-coat) is ade2. Consequently, it accumulates a metabolite, resulting in *pink-stained colonies*. The plasmid coding the bridging RNA harbors the ADE2 gene that restores the white phenotype. This allows for selection of transformants where plasmid coding the bridging RNA is necessary for reporter transcription.

panel), is able to produce a supershifted complex, indicating AUF1 binding. By contrast, when the *bcl-2* ΔARE riboprobe (in which the 107-nt ARE core was deleted) was used, supershift did not occur, indicating no AUF1 binding. Other REMSA experiments have been carried out with each AUF1 isoform (p37, p40, p42, and p45) synthesized *in vitro*. As shown in Fig. 3, all AUF1 isoforms shift *bcl-2* ARE+ and *bcl-2* ARE riboprobes but not the *bcl-2* ΔARE riboprobe, indicating that binding of the *bcl-2* mRNA to AUF1 requires the ARE core.

To evaluate the possibility that mRNA destabilizing activity and AUF1 binding of the *bcl-2* ARE are two related events, analysis of mRNA half-life in cells expressing a reporter gene with or without the *bcl-2* ARE was performed. For this purpose, three NIH 3T3 cell lines stably expressing a rabbit β-globin gene transcriptionally driven by the c-*fos* serum-inducible promoter with either *bcl-2* ARE+ (pBBB-U1) or the ARE-deleted *bcl-2* ΔARE (pBBB-U2) or without insert (pBBB4) were used. Following serum addition to induce a pulse of transcription of each reporter gene, total RNA was extracted at various times, and β-globin mRNA levels were quantitated by RNase protection using glyceraldehyde-3-phosphate dehydrogenase as an internal standard (Fig. 4). With respect to the β-globin transcript without insert (BBB4), the insertion of *bcl-2* ARE+ (BBB-U1), which binds AUF1, reduces the half-life of reporter mRNA by 6-fold (from >12 h to 2 h). The insertion of *bcl-2* ΔARE (BBB-U2), which does not bind AUF1, does not affect the half-life of β-globin mRNA.

All these results clearly indicate that the 107 nt of the ARE of *bcl-2* (shared by *bcl-2* ARE and *bcl-2* ARE+), but not its flanking regions (*bcl-2* ΔARE), contain the binding site for AUF1 and are also required for mRNA destabilizing activity. Thus, we have used the *bcl-2* ARE as a riboprobe in further experiments.

We assumed that the smallest isoform, p37, was the prototype of AUF1 because its entire sequence, harboring two RNA

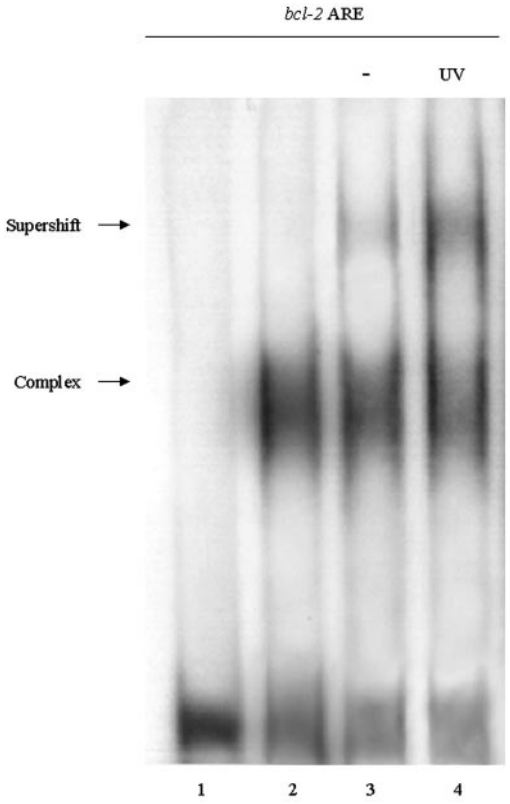


FIG. 6. **Effect of UVC irradiation on AUF1 (REMSA/supershift).** *Lane 1*, *bcl-2* ARE riboprobe; *lane 2*, *bcl-2* ARE riboprobe incubated with cytoplasmic extracts; *lane 3*, *bcl-2* ARE riboprobe incubated with cytoplasmic extracts and anti-AUF1 Ab; *lane 4*, *bcl-2* ARE riboprobe incubated with cytoplasmic extracts from UVC-treated cells irradiated for 8 h and anti-AUF1 Ab. *Lane 1* indicates free RNA digested with RNase T1. Complexes and supershifts are indicated.

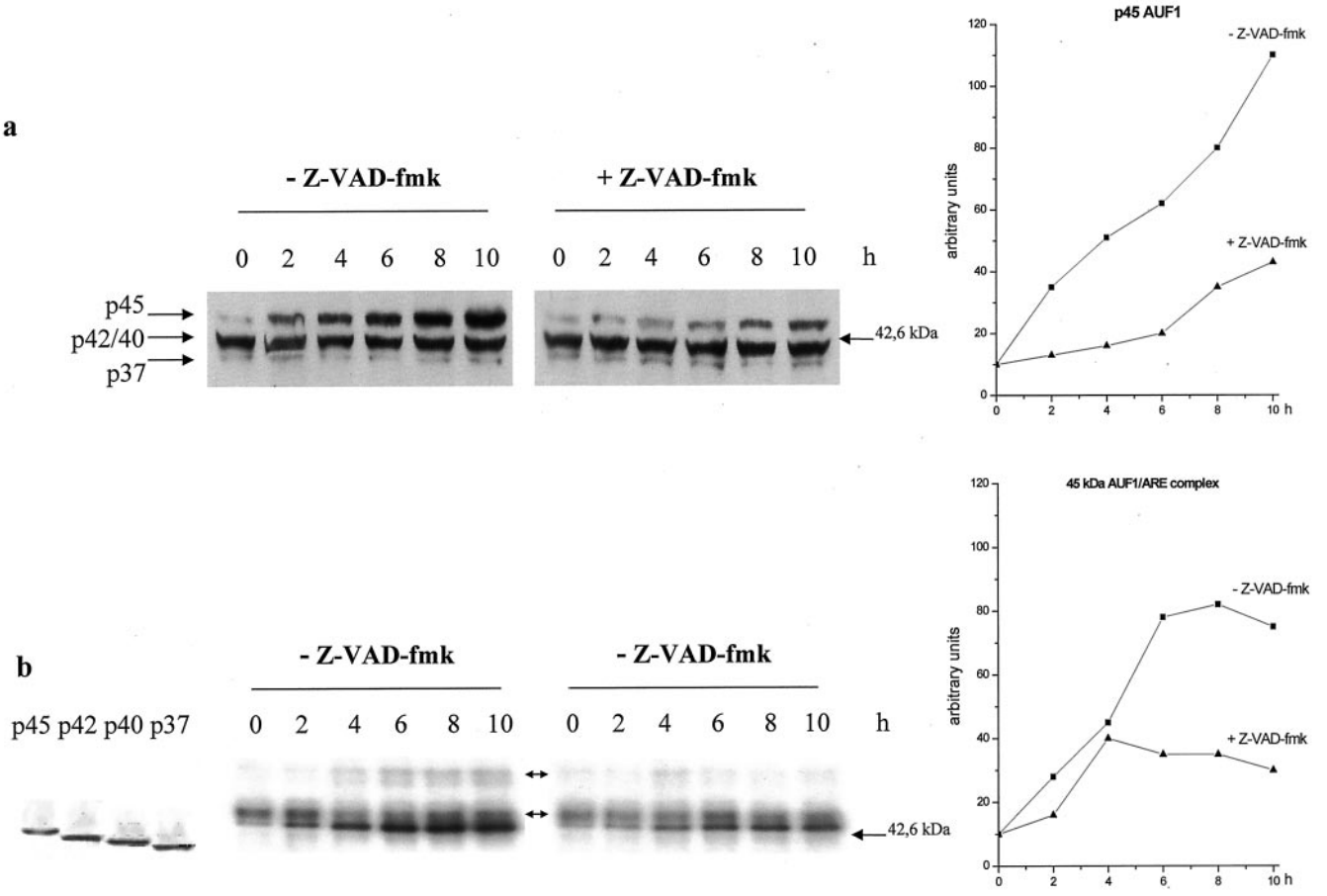


FIG. 7. **Effect of UVC irradiation on AUF1 levels and *bcl-2* ARE/AUF1 binding.** Western blots (*a*) and immunoprecipitations (*b*) were carried out with cytoplasmic extracts from Jurkat cells obtained at various time points after UVC irradiation that had been pretreated or not pretreated with Z-VAD-fmk, as indicated. In Western blot analysis, immunodetection and molecular mass markers run in the same gel were used to identify each AUF1 isoform. In immunoprecipitation experiments, the extracts were cross-linked with the *bcl-2* ARE riboprobe before incubation with the anti-AUF1 Ab. The molecular mass of the complexes was evaluated on the basis of labeled, *in vitro*-synthesized AUF1 isoforms and molecular mass markers run in the same gel. *Double-headed arrows* in *b* indicate other complexes with higher molecular masses than the 45-kDa complex. Quantitations of Western blots and immunoprecipitations are shown to the *right* in each panel.

recognition motifs, is contained in the other three isoforms. The yeast RNA THS was used to demonstrate that recombinant p37 binds to the ARE of *bcl-2 in vivo* (Fig. 5). The reporter gene activation of the host yeast was tested in parallel with the indicated positive and negative controls. The 1–257-amino acid segment of p37 containing the two RNA recognition motifs of AUF1 elicited the activation of both LacZ and HIS3 genes, clearly demonstrating that binding of AUF1 to the *bcl-2* ARE also occurs *in vivo* (Fig. 5).

An Increase of AUF1 and the bcl-2 mRNA-AUF1 Complex Are Associated with UVC-induced Apoptosis and Are Partially Prevented by the Caspase Inhibitor Z-VAD-fmk—The possibility that enhanced decay of *bcl-2* mRNA during apoptosis (8, 9) is associated with changes of AUF1 levels was evaluated. Jurkat cells were irradiated with UVC at a dose previously established to commit cells to apoptosis (data not shown). REMSA experiments demonstrated an increase of the supershifted AUF1-*bcl-2* ARE complex at 8 h compared with no UVC (Fig. 6, compare *lane 3* with *lane 4*). To establish the involvement of the AUF1 isoforms in the increase of supershifted complex, we carried out time course Western blot analyses and immunoprecipitation experiments of UV-cross-linked complexes with anti-AUF1 Ab (Fig. 7, *a* and *b*, respectively). Each AUF1 isoform was identified by immunodetection and comparison with standard molecular mass markers. During the time course (0–10 h) after apoptotic stimulation (Fig. 7*a*), the cytoplasmic p45 isoform markedly increased within 2 h after UVC irradiation, peaking at 10 h. Pretreatment with Z-VAD-fmk attenuated this increase by 2–3-fold. The increase of p37 and p40/p42 isoforms after UVC irradiation was very low and was not affected by Z-VAD-fmk pretreatment. To determine whether the AUF1 variations observed during apoptosis are accompanied by an increase of some isoform-specific ARE binding activity, immunoprecipitation analyses of UV-cross-linked complexes were performed (Fig. 7*b*). Standard molecular mass markers and labeled AUF1 isoforms translated *in vitro* and run in the same gel were used for comparison. During the time course, the AUF1 isoform-*bcl-2* ARE complex with an apparent molecular mass of about 45 kDa underwent a marked increase, which paralleled the increase of p45 AUF1 (Fig. 7*a*) in Western analysis and, analogously, is attenuated by pretreatment with Z-VAD-fmk. Another complex with lower molecular mass (about 37 kDa) was detected as a very faint band. No other complexes have been detected within the molecular mass range of the AUF1 isoforms. The additional bands with higher molecular masses observed in the immunoprecipitate (indicated by *double-headed arrows*) may represent AUF1 isoform dimers or multimers stabilized by UV-cross-linking. From all described results, we conclude that AUF1 is a *bcl-2* AUBP and that an isoform-specific mechanism is involved in ARE-mediated *bcl-2* mRNA decay during apoptosis. Furthermore, our results suggest that the p45 AUF1 isoform is the most likely candidate to play a pivotal role in this mechanism.

DISCUSSION

bcl-2 is one of the most studied apoptosis-related genes and is also implicated in cell cycle progression and cell differentiation. The expression of *bcl-2* can be finely tuned by a variety of environmental or endogenous stimuli and regulated at both transcriptional (5, 33) and posttranscriptional levels (8). The diverse modes of regulation of *bcl-2* expression probably reflect different requirements for down- or up-regulation in different physiological conditions or in pathological processes.

We described a posttranscriptional level of regulation of *bcl-2* expression that is mediated by an ARE in the 3′-UTR of *bcl-2* mRNA. Under normal conditions, this element has a moderate destabilizing activity toward the *bcl-2* transcript. This activity increases following apoptotic stimuli, leading to enhanced *bcl-2* mRNA degradation (8). The *bcl-2* mRNA ARE binds to a number of AUBPs whose electrophoretic pattern changes after induction of apoptosis (9). A number of AUBPs that modulated mRNA stability have been described for other ARE-containing mRNAs such as *VEGF* (37, 38), *GM-CSF* (39, 40), and c-*fos* (15, 41, 42). We first demonstrated that AUF1 is a *bcl-2* AUBP involved in *bcl-2* down-regulation during apoptosis. AUF1 is a heterogeneous nuclear ribonucleoprotein protein implicated in mRNA stability regulation, and it shuttles dynamically between the nucleus and cytoplasm (43). Although the four isoforms of AUF1, namely, p45, p42, p40, and p37 (29, 30), are present in both the nucleus and cytoplasm of Jurkat cells (data not shown), all our experiments have been carried out using cytoplasmic extracts because the cytoplasm is the compartment of mRNA degradation. The binding of AUF1 to the ARE of *bcl-2* was demonstrated *in vitro* by means of supershift REMSA experiments. The cytoplasmic extracts were incubated simultaneously with *bcl-2* ARE radiolabeled riboprobes and either anti-AUF1 or anti-TK Ab. In these experiments, only the specific anti-AUF1 Ab was able to supershift the *bcl-2* ARE-protein complex. All AUF1 isoforms (p37, p40, p42, and p45) have the potential to bind to the *bcl-2* mRNA, as indicated *in vitro* by results obtained in REMSA experiments carried out with each single *in vitro*-synthesized isoform. Using p37, considered as the AUF1 prototype, in the yeast RNA THS we demonstrated that the AUF1 RNA binding motifs common to all four isoforms are also able to bind to the *bcl-2* ARE *in vivo*.

Deletion of the evolutionary conserved ARE segment particularly rich in AU motifs (and therefore considered as the ARE core) not only abolishes the ability of the *bcl-2* ARE to bind AUF1 but also abolishes its mRNA destabilizing activity. This indicates that the deleted segment harbors the binding site for AUF1 and that AUF1 is required as a trans-acting factor for functional activity of the *bcl-2* ARE.

The implication of AUF1 as a trans-acting factor involved in ARE-mediated, *bcl-2* mRNA degradation during apoptosis was further demonstrated by results obtained in supershift REMSA experiments carried out after UVC irradiation of cultured cells. Furthermore, we found that the level of the p45 isoform, as evaluated by Western blot, increased markedly until 10 h after UVC irradiation. In the same extracts, the level of one AUF1-*bcl-2* ARE complex paralleled the increased level of p45 AUF1, and its molecular mass was also about 45 kDa. This complex was also the only one to undergo a clear decrease in Z-VAD-fmk-treated cells compared with untreated cells. This decrease also parallels the decrease in the 45-kDa isoform observed by Western blot of extracts from cells pretreated with Z-VAD-fmk with respect to untreated cells. Whereas these data are suggestive, the specific AUF1 isoform(s) involved in *bcl-2* ARE binding and mRNA degradation during apoptosis could not be precisely identified on the basis of its molecular mass. Nevertheless, our results indicate that at least one AUF1 isoform is a candidate to play a pivotal role in this regulatory mechanism.

Arao *et al.* (43) found that p45 and p42 are predominantly nuclear proteins and that, consequently, the observed increase, if actually attributable to one of these isoforms, could be explained by nuclear to cytoplasm shuttling. This possibility is supported by our previous observation that the apoptotic stimulus by cycloeximide (50 μM), utilized to block protein synthesis, resulted in an analogous increase in *bcl-2* ARE-bound, cytoplasmic AUBPs (9). In our model, the relative balance of the AUF1 isoforms seems to determine the fate of the *bcl-2* mRNA in response to apoptotic stimuli. We speculate that this balance could be affected in response to other types of endogenous and environmental stimuli. Results obtained from Western blot as well as immunoprecipitation assays suggest that binding of AUF1 isoforms to *bcl-2* mRNA in cellular extracts could also depend on the other amino acid sequence motifs by which the isoforms differ.

We also speculate that the scarce signal of the relatively low molecular mass complex (about 37 kDa), the absence of other complexes in the range of the AUF1 molecular masses, and the presence of higher molecular mass complexes in immunoprecipitates could result from the AUF1 isoform ability to multimerize as reported by Wilson *et al.* (44). Our results also strengthen the hypothesis that the various isoforms of AUF1 may reflect the need of cells to differentially modulate ARE-containing mRNAs during apoptosis. Furthermore, mRNA turnover of ARE-containing genes has already been demonstrated to be a finely tuned process. For example, Wang *et al.* (24) demonstrated that the ARE-containing mRNA of $p21^{waf1}$, a p53-inducible gene responsible for cell cycle inhibition upon UVC irradiation, was stabilized by the AUBP HuR. This would account for the arrest of cell growth that occurs at the first stages of apoptosis. Other conditions, such as hemin-induced erythroid differentiation, are able to impair ARE activity by sequestration of AUF1 into a complex of proteins (45).

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AUF1 Is a *bcl-2* A + U-rich Element-binding Protein Involved in *bcl-2* mRNA Destabilization during Apoptosis

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