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Regulation of Bcl-2 Expression by HuR in HL60 Leukemia Cells and A431 Carcinoma Cells

Daniella Ishimaru,[1] Sivakumar Ramalingam,[1] Tapas K. Sengupta,[1] Sumita Bandyopadhyay,[1] Stephanie Dellis,[2] Baby G. Tholanikunnel,[1] Daniel J. Fernandes,[1] and Eleanor K. Spicer[1]

[1]*Department of Biochemistry and Molecular Biology, Medical University of South Carolina and*
[2]*Department of Biology, College of Charleston, Charleston, South Carolina*

Abstract

Overexpression of the proto-oncogene *bcl-2* promotes abnormal cell survival by inhibiting apoptosis. Expression of *bcl-2* is determined, in part, by regulatory mechanisms that control the stability of *bcl-2* mRNA. Elements in the 3′-untranslated region of *bcl-2* mRNA have been shown to play a role in regulating the stability of the message. Previously, it was found that the RNA binding proteins nucleolin and Ebp1 have a role in stabilizing *bcl-2* mRNA in HL60 cells. Here, we have identified HuR as a component of *bcl-2* messenger ribonucleoprotein (mRNP) complexes. RNA coimmunoprecipitation assays showed that HuR binds to *bcl-2* mRNA *in vivo*. We also observed an RNA-dependent coprecipitation of HuR and nucleolin, suggesting that the two proteins are present in common mRNP complexes. Moreover, nucleolin and HuR bind concurrently to *bcl-2* AU-rich element (ARE) RNA *in vitro*, suggesting separate binding sites for these proteins on *bcl-2* mRNA. Knockdown of HuR in A431 cells leads to down-regulation of *bcl-2* mRNA and protein levels. Observation of a decreased ratio of *bcl-2* mRNA to heterogeneous nuclear RNA in HuR knockdown cells confirmed a positive role for HuR in regulating *bcl-2* stability. Recombinant HuR retards exosome-mediated decay of *bcl-2* ARE RNA in extracts of HL60 cells. This supports a role for HuR in the regulation of *bcl-2* mRNA stability in HL60 cells, as well as in A431 cells. Addition of nucleolin and HuR to HL60 cell extracts produced a synergistic protective effect on decay of *bcl-2* ARE RNA. HuR knockdown also leads to redistribution of *bcl-2* mRNA from polysomes to monosomes. Thus, HuR seems to play a positive role in both regulation of *bcl-2* mRNA translation and mRNA stability. (Mol Cancer Res 2009;7(8):1354–66)

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Current address for T.K. Sengupta: School of Biology, Indian Institute of Science, Education and Research, Kolkata 700106, India. Current address for S. Bandyopadhyay: Department of Biophysics, Molecular Biology and Bioinformatics, University of Kolkata, 700009 Kolkata, India.
Requests for reprints: Eleanor K. Spicer, Department of Biochemistry and Molecular Biology, Medical University of South Carolina, P.O. Box 250509, Charleston, SC 29425. Phone: 843-792-7475; Fax: 843-792-8565. E-mail: spicer@musc.edu
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Introduction

Bcl-2 protein belongs to a family of proteins that are fundamentally important in the regulation of apoptosis and tumorigenesis. In fact, *bcl-2* was one of the first proto-oncogenes found to promote carcinogenesis by prolonging cell survival rather than by increasing the rate of cell proliferation (1). Bcl-2 protein seems to exert its antiapoptotic activity by heterodimerizing with its proapoptotic partners BAX and BAK (2, 3), whereas its activity can be down-regulated by interaction with other proteins, such as the proapoptotic BAD protein (4). Therefore, it has been suggested that the balance of the levels of Bcl-2 family members is an important factor in determining cell fate (5). Accordingly, the level of *bcl-2* expression is a critical determinant of the sensitivity of cells to apoptotic stimuli. Not surprisingly, a number of cancers have been described to have high levels of Bcl-2 protein (6-8), and elevated *bcl-2* expression has been found to contribute to resistance to chemotherapeutic agents of some hematologic malignancies (9, 10).

In human lymphomas, t(14:18) translocations have been observed that produce significant transcriptional activation of *bcl-2* expression (11). However, high-level expression of *bcl-2* mRNA and protein is often seen in chronic lymphocytic leukemias (8) and breast cancers (6, 12) where there is no evidence for gene rearrangements or promoter mutations that are known to enhance *bcl-2* transcription (7, 13-16). In these cancers, the overexpression of Bcl-2 protein seems to be, at least in part, a consequence of increased *bcl-2* mRNA stability (8, 12).

mRNA stability is governed by orchestrated interactions between sequence and/or structural elements (*cis* elements) in mRNAs and specific *trans*-acting factors that recognize these elements. The best-characterized *cis*-acting sequences responsible for mRNA decay in mammalian cells are the AU-rich elements (ARE) present in the 3′-untranslated region (3′-UTR) of short-lived mRNAs (17). Interestingly, there is a class II ARE (17) present in the 3′-UTR of *bcl-2* mRNA ($ARE^{bcl\text{-}2}$) that plays a role in regulating the levels of *bcl-2* message within a variety of cells. We (18) and others (19) have reported that the $ARE^{bcl\text{-}2}$ exhibits destabilizing activity when fused to the *β-globin* gene in transfected NIH 3T3 cells. Schiavone and coworkers (19) found that the decay of *β-globin*-$ARE^{bcl\text{-}2}$ transcripts was enhanced by treatment of transfected cells with the apoptosis agent ceramide, suggesting that the ARE motif is physiologically relevant to the regulation of *bcl-2* expression by apoptotic agents (20). Subsequently, it was found that the association of ARE binding proteins with the $ARE^{bcl\text{-}2}$ was modulated by UVC irradiation (20). Additionally, we have observed that treatment of human leukemia HL60 cells with Taxol or okadaic acid leads to decreased

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binding of cytoplasmic factors to ARE$^{bcl\text{-}2}$ RNA (18) and that treatment with retinoic acid leads to destabilization of *bcl-2* mRNA (21, 22).

Previously, we identified nucleolin as one of the proteins that bind to and stabilize *bcl-2* mRNA in HL60 cells (23). We have shown that Taxol- or okadaic acid–induced destabilization of *bcl-2* mRNA occurs through a process that involves down-regulating the activity of nucleolin. Subsequently, we found that the human proliferation-associated protein Ebp1 also is a *bcl-2* mRNA stabilizing factor (24). However, earlier UV-cross-linking studies (18) suggested that as many as four to six proteins in HL60 cells may interact with *bcl-2* mRNA and play a role in regulating *bcl-2* mRNA stability. The goal of the current work was to determine if the RNA binding protein HuR is one of the previously unidentified proteins that have a role in regulating *bcl-2* expression.

HuR is one of the most well-studied ARE-binding proteins (25, 26). It binds ARE sequences with high affinity (27), but its role in mRNA regulation seems to vary depending on its target. For instance, HuR has been described to increase the stability of *cox-2, cyclin D1, oncostatin M*, and *p21* mRNAs, among others (28-31). On the other hand, HuR has been found not only to increase the translational efficiency of *p53* mRNA (32) but also to decrease translation of *TNF*α mRNA (33). Moreover, high cytoplasmic levels of HuR have been associated with higher tumor grade, increased cyclooxygenase-2 expression, and poor survival rates in breast carcinoma (34, 35), suggesting a role for this protein in cancer pathogenesis.

Recently, Abdelmohsen et al. (36) reported that HuR protein interacts with *bcl-2* mRNA in unstimulated HeLa cells. Knockdown of HuR was associated with decreased *bcl-2* mRNA levels and decreased Bcl-2 protein levels. However, it was not known if decreased *bcl-2* mRNA levels were due to changes in *bcl-2* mRNA stability or indirect effects on *bcl-2* transcription. Additionally, it was uncertain whether HuR also affected translation of *bcl-2* mRNA in HeLa cells. mRNA stabilization by *trans*-acting factors is cell specific and often involves interactions of multiple factors with a single mRNA. Thus, the findings of Abdelmohsen and coworkers (36) raise the additional question of whether HuR also plays a role in regulating *bcl-2* expression in HL60 leukemia cells, where Bcl-2 protein is highly expressed (7). To address these questions, the activities of HuR in promoting *bcl-2* mRNA stability and translation efficiency were examined.

Results

Identification of HuR in ARE$^{bcl\text{-}2}$ *RNA Complexes*

We have previously showed that nucleolin binds to the ARE$^{bcl\text{-}2}$ (nucleotides 1383-1519 of *bcl-2* mRNA; NM_000633) and plays a role in the regulation of *bcl-2* mRNA stability (23, 24). Earlier UV cross-linking assays suggested that additional, previously unidentified proteins of 30 to 55 kDa bind to *bcl-2* mRNA in HL60 cells (18). One likely candidate protein is the 36-kDa mRNA binding protein HuR. To examine the possibility that HuR binds to *bcl-2* mRNA, we tested whether or not immunoprecipitation of nucleolin leads to coprecipitation of HuR. Cytoplasmic extracts of HL60 cells were preincubated with either RNase inhibitor or RNase I. Treated extracts were then incubated with IgG or anti-nucleolin antibody. Recovered immunoprecipitates were analyzed for the presence of HuR or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by Western blot analysis. As shown in Fig. 1, Western blots of anti-nucleolin–precipitated complexes showed the presence of HuR in extracts treated with RNase inhibitor. Interestingly, pretreatment of cell extracts with RNase I greatly reduced the amount of HuR recovered in the anti-nucleolin immunoprecipitates. This suggests that the association of HuR and nucleolin is dependent on the presence of RNA, consistent with the concept that nucleolin and HuR are present in common messenger ribonucleoprotein (mRNP) complexes. Although GAPDH is an mRNA binding protein, no GAPDH was detected in the precipitates (Fig. 1), indicating that the RNA-mediated interaction between HuR and nucleolin is specific.

To further test whether HuR interacts with *bcl-2* mRNA in HL60 cells, gel mobility supershift assays were done. ARE$^{bcl\text{-}2}$ RNA transcripts labeled with [^{32}P]UTP were incubated with S10 cytoplasmic extracts of HL60 cells in the absence of antibodies or in the presence of anti-HuR antibody, anti-nucleolin antibody, or IgG antibody. RNA/protein/antibody complexes were separated from RNA/protein complexes on an agarose gel. Figure 2A shows that incubation of ARE$^{bcl\text{-}2}$ RNA with HL60 cytoplasmic extracts produced a protein/ARE$^{bcl\text{-}2}$ RNA complex (compare *lanes 1* and *5*). Addition of anti-HuR antibody produced a shift of the mobility of the protein/ARE$^{bcl\text{-}2}$ complex (i.e., produced a supershifted complex; *lane 3*). Incubation of antibody with the RNA transcript before addition of cell extract produced a similar supershifted complex, indicating that the order of addition of reactants does not affect the formation of the supershifted complex (*lane 4*). No supershift was observed with IgG antibodies (*lane 2*). As expected, addition of anti-nucleolin antibody also produced a shift in the protein/RNA complex (*lane 6*). This indicates that both HuR and nucleolin are present in ARE$^{bcl\text{-}2}$ RNA/protein complexes that are formed with cytoplasmic extracts of HL60 cells.

HuR Binds to bcl-2 mRNA In vivo *in HL60 Cells*

To determine if the HuR-*bcl-2* mRNA interactions observed above also occur *in vivo*, RNA immunoprecipitation assays were done. Because protein-RNA interactions detected in cell lysates sometimes do not reflect what occurs *in vivo* (37), immunoprecipitations were done with extracts from cross-linked cells. Protein/nucleic acid complexes within HL60 cells were cross-linked by formaldehyde treatment (38) and total cell extracts were incubated with anti-HuR, anti-CD55, or IgG antibodies. Before RNA purification, recovered immunoprecipitated complexes were heat-treated to reverse protein-nucleic acid cross-links. The extracted RNA was examined for the presence of *bcl-2* mRNA by real-time quantitaive PCR (qRT-PCR). As shown in Fig. 2B, *bcl-2* mRNA was recovered from anti-HuR precipitates whereas only trace amounts were obtained from complexes precipitated with IgG antibody. In addition, precipitation with antibody to CD55 (39), a non-mRNA binding protein, did not bring down *bcl-2* mRNA. *GAPDH* mRNA was not appreciably recovered in precipitates with any of the three antibodies. In reactions where extracts were pretreated with RNase, little *bcl-2*

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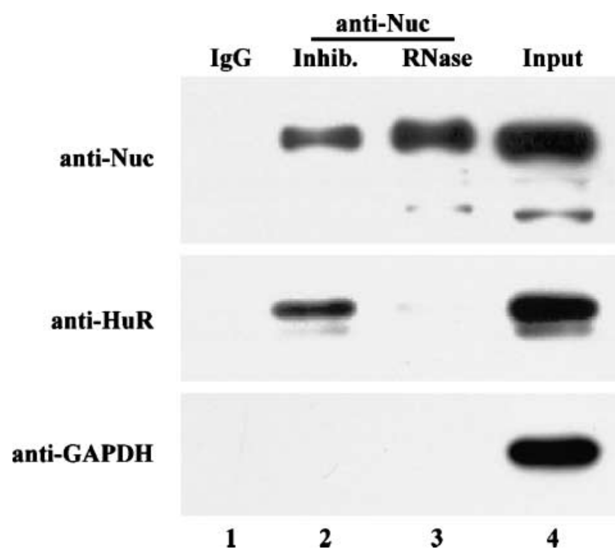

FIGURE 1. Coimmunoprecipitation of HuR and nucleolin. S10 cytoplasmic extracts of HL60 cells were preincubated with RNase inhibitor or RNase I. Extracts were then incubated with anti-nucleolin or IgG antibodies, and protein/antibody complexes were isolated by incubation with protein A/G-agarose beads. Western blot analysis of the precipitated complexes was done with anti-HuR, anti-nucleolin, or anti-GAPDH antibodies, as indicated. Lane 1, pull-down with IgG in extracts treated with RNase inhibitor; lane 2, pull-down with anti-nucleolin antibody in extracts treated with RNase inhibitor; lane 3, pull-down with anti-nucleolin in extracts with RNase I; lane 4, a fraction of the S10 extract (input control).

mRNA was recovered. Collectively, these results indicate that HuR binds specifically to *bcl-2* mRNA *in vivo* in HL60 cells.

Recombinant HuR Binds to bcl-2 *RNA Transcripts* In vitro

The above assays (Fig. 2A and B) indicated that HuR binds to *bcl-2* mRNA *in vitro* and *in vivo*. To determine if HuR binds directly to *bcl-2* mRNA rather than binding through interactions with another protein, recombinant HuR was purified and tested for *bcl-2* mRNA binding activity. A fusion protein containing a His tag and full-length human HuR was expressed from plasmid pET21a-HuR in *E. coli* and was purified to ≥90% purity, as described in Materials and Methods. Binding of HuR-His to a 200-nucleotide ARE^{bcl-2} transcript was examined by RNA gel mobility shift assays. As shown in Fig. 3A, incubation of recombinant HuR with ARE^{bcl-2} RNA produced a shift in the RNA mobility. In contrast, HuR did not form a complex with a β-globin RNA transcript (Fig. 3A). Under native gel conditions, both ARE^{bcl-2} and β-globin transcripts migrate as two bands (Fig. 3A), presumably corresponding to two conformations of the RNA, because under denaturing conditions, a single band is seen (see Fig. 7). Interestingly, HuR binds both conformations of ARE^{bcl-2}. As shown in Fig. 3A, multiple complexes with ARE^{bcl-2} RNA are formed in the presence of increasing concentrations of HuR, indicating that more than one HuR can bind to the ARE^{bcl-2} transcript. This is in agreement with the observations of Fialcowitz-White et al. (27) that HuR forms oligomeric complexes with ARE substrates. Based on the concentrations of HuR at which half of the free RNA is bound, the apparent K_d for binding of HuR to ARE^{bcl-2} is estimated to be ≈80 nmol/L. However, this is likely an underestimation of the affinity because it does not take into account the oligomeric complexes of HuR associated with ARE^{bcl-2} RNA.

To identify the binding site of HuR on ARE^{bcl-2}, the affinity of HuR for fragments of the ARE^{bcl-2} was assessed. Four contiguous fragments of ARE^{bcl-2} (Fig. 3B) were chemically synthesized and tested for HuR binding. As shown in Fig. 3C, HuR binds to fragments B and C with modest affinity (K_d ≈ 400 nmol/L). However, HuR showed little or no affinity for fragment A or D. Thus, the primary binding site for HuR seems to be in the middle of the ARE.

HuR and Nucleolin Bind Concurrently to ARE^{bcl-2} *RNA* In vitro

Our previous studies have shown that nucleolin binds to ARE^{bcl-2} RNA and increases *bcl-2* mRNA stability *in vitro* (23) and *in vivo* (12). In addition, as shown in Fig. 1, nucleolin and HuR are able to simultaneously bind to a common RNA. Therefore, to determine whether or not nucleolin and HuR bind to distinct nonoverlapping sites on the ARE^{bcl-2}, RNA gel mobility shift assays were done in the presence of both proteins. Figure 4 shows a representative gel-shift assay in which ^{32}P-ARE^{bcl-2} RNA (*lanes 1* and *10*) was incubated with nucleolin alone (*lane 2*), with nucleolin and increasing concentrations of GST-HuR (*lanes 3-5*), with GST-HuR alone (*lane 6*), or with GST-HuR and increasing concentrations of nucleolin (*lanes 7-9*). This assay shows that incubation of ARE^{bcl-2} RNA with excess amounts of either nucleolin or GST-HuR alone produces a single RNA/protein complex (*lanes 2* and *6*, respectively). Incubation of GST polypeptide with ARE^{bcl-2} RNA did not produce a shift (Supplementary Fig. S1). Interestingly, the addition of HuR to reactions containing nucleolin/ARE^{bcl-2} RNA complexes produced a complex that had a slower migration rate than the nucleolin/ARE^{bcl-2} RNA complexes (*lanes 3-5*). Reciprocally, addition of nucleolin to reactions containing HuR produced an intermediate-mobility complex similar to the complex formed when HuR was added to reactions preincubated with nucleolin (*lanes 7-9*). The intermediate migration rate of the complexes (i.e., between the fast migration complex with only nucleolin and the slow migration complex with only HuR) suggests that they contain both HuR and nucleolin. The in-between migration may also indicate that binding of either protein blocks saturating binding by the other protein. This could occur from binding of the two proteins to adjacent sites or from the induction of RNA conformational changes by one or both proteins. In either case, this assay provides evidence that HuR and nucleolin can bind concurrently to ARE^{bcl-2} RNA *in vitro*.

Short Hairpin RNA–Mediated Knockdown of HuR Reduces the Levels of bcl-2 *mRNA and Protein*

HuR protein has been found to stabilize the mRNA of a number of genes, including *c-fos, p21, GM-CSF*, and *VEGF* (reviewed in ref. 40). However, in some cases, HuR has been found to modulate translation rather than the stability of the mRNA (32, 33). To determine the role of HuR in the regulation of *bcl-2* mRNA, HuR knockdown studies were done. Repeated efforts to obtain stable knockdown of HuR expression in HL60 cells were unsuccessful. As an alternative, short hairpin RNA (shRNA) knockdown of HuR was done in human

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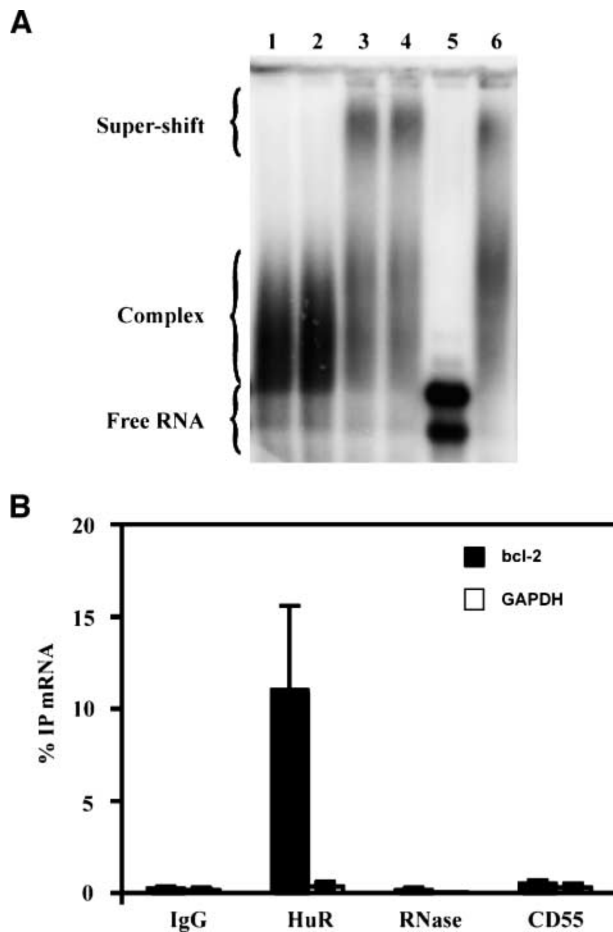

FIGURE 2. Detection of HuR-*bcl-2* mRNA interactions. **A.** ^{32}P-ARE$^{bcl\text{-}2}$ RNA was incubated without (*lane 5*) or with (*lanes 1-4* and *6*) S10 cytoplasmic extract from HL60 cells. The presence of HuR and nucleolin in protein/RNA complexes was tested by addition to the reaction of anti-HuR antibody (*lanes 3* and *4*), control IgG antibody (*lane 2*), or anti-nucleolin antibody (*lane 6*). Samples were separated on a 1.2% agarose gel, which was dried and analyzed by phosphorimaging. In lane 4, RNA was incubated with anti-HuR antibody for 10 min on ice before addition of S10 extract. **B.** Whole-cell lysates from formaldehyde-cross-linked HL60 cells were incubated with anti-HuR, anti-CD55, or IgG antibodies and RNA/protein/antibody complexes were recovered by incubation with protein A/G-agarose beads. The presence of *bcl-2* and *GAPDH* mRNAs in immunoprecipitated complexes was detected by qRT-PCR. Negative control reactions were done with IgG or with extracts pretreated with RNase and immunoprecipitated with anti-HuR antibody. Representative of the findings in three experiments.

A431 epithelial cancer cells. A431 cells were transfected with a plasmid expressing HuR shRNA or scrambled shRNA and G418-resistant stable transfectants were selected. qRT-PCR analysis revealed that *HuR* mRNA was reduced in HuR shRNA–transfected cells to ~30% of the level found in cells transfected with a scrambled shRNA (Fig. 5A). Western blot analysis indicated that HuR protein levels also were greatly reduced in the HuR shRNA–transfected cells (97% reduction; Fig. 5B). In contrast, β-tubulin protein levels were similar in cells transfected with HuR shRNA or scrambled shRNA. Importantly, qRT-PCR assays showed that *bcl-2* mRNA levels were reduced to ~60% in shHuR transfectants relative to the levels of *bcl-2* mRNA in cells transfected with a scrambled shRNA (Fig. 5C). Additionally, Western blot analysis showed that Bcl-2 protein levels were much lower in HuR shRNA knockdown cells than in control cells (96% reduction; Fig. 5D). The finding that *bcl-2* mRNA levels are reduced in HuR knockdown cells suggests that HuR has a role in regulating *bcl-2* mRNA stability. The finding that HuR knockdown had a greater effect on Bcl-2 protein levels than on *bcl-2* mRNA levels suggests that HuR has a positive role in translation of *bcl-2* mRNA.

To confirm that the reduction in *bcl-2* mRNA level was a result of reduced mRNA stability, the levels of *bcl-2* heterogeneous nuclear RNA (hnRNA; newly synthesized unspliced transcript) and *bcl-2* mature mRNA were measured in HuR shRNA– and scrambled shRNA–transfected cells. This approach has been used successfully to measure the relative rate of mRNA decay and mRNA transcription in a variety of cells (41, 42). This method was used in preference to measuring mRNA levels following treatment of cells with actinomycin D because this drug has been found to affect the stability of some mRNAs that contain ARE motifs (43). Additionally, actinomycin D was found to cause translocation of HuR from the nucleus to the cytoplasm (44). This could cause stabilization of *bcl-2* mRNA in the cells transfected with scrambled shRNA, leading to overestimation of stabilization of *bcl-2* mRNA. In comparing hnRNA and mRNA levels, if the decrease in *bcl-2* mRNA levels in HuR shRNA–transfected cells is primarily due to mRNA destabilization rather than changes in transcription, then the ratio of mRNA to hnRNA would be lower in HuR shRNA–transfected cells compared with scrambled shRNA. Figure 6 shows *bcl-2* mRNA and hnRNA levels relative to *RPL13a* mRNA in cells transfected with HuR shRNA and scrambled shRNA. The finding that the ratio of mRNA to hnRNA in HuR shRNA–transfected cells is lower than that in the scrambled shRNA–transfected cells indicates that knockdown of HuR results in decreased *bcl-2* mRNA stability.

HuR Retards ARE$^{bcl\text{-}2}$ *RNA Decay* In vitro

To determine the potential of HuR as a modulator of *bcl-2* mRNA stability in HL60 cells, the effect of recombinant HuR on *bcl-2* mRNA decay in HL60 S100 cytoplasmic cell extracts was examined. RNA decay assays were done using the *in vitro* system developed by Ford and Wilusz (45) and Mukherjee et al. (46). For these assays, 5′-^{7}mGpppG–capped, nonpolyadenylated ARE$^{bcl\text{-}2}$ and β-globin transcripts were synthesized *in vitro*. In this cell-free system, RNAs are processively shortened by 3′-5′ exosome degradation rather than decapping and 5′-3′ degradation (46). To test for exosomal degradation, we examined the rate of decay of ARE$^{bcl\text{-}2}$ and *β-globin* transcripts in soluble (S100) extracts of HL60 cells. At various times after addition to cell extracts, RNA was recovered and analyzed by gel electrophoresis. The observation that ARE$^{bcl\text{-}2}$ transcripts decayed faster ($t_{1/2}$ = 16 minutes) than *β-globin* transcripts ($t_{1/2}$ = 46 minutes; compare Fig. 7A and B, densitometry in Fig. 7C) is consistent with decay being exosome mediated because the exosome has been shown to enhance the rate of decay of ARE-containing mRNAs *in vitro* (46). As expected, decay was processive, as no intermediate sized products were observed on the gels (Fig. 7A, B, and D).

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To examine the effects of HuR on ARE$^{bcl\text{-}2}$ stability, the rate of decay of ARE$^{bcl\text{-}2}$ transcripts was measured in HL60 cell extracts supplemented with exogenous HuR protein. As shown in Fig. 7A and E, decay of ARE$^{bcl\text{-}2}$ RNA was considerably slower in the presence of recombinant HuR protein ($t_{1/2}$ = 42 minutes) than in unsupplemented S100 extracts ($t_{1/2}$ = 16 minutes), which have very low amounts of endogenous HuR (Supplementary Fig. S2). Addition of bovine serum albumin (BSA) to cell extracts had little effect on the rate of decay of ARE$^{bcl\text{-}2}$ RNA ($t_{1/2}$ = 17 minutes; Fig. 7D and E). These findings show that HuR can block exosome degradation of *bcl-2* transcripts *in vitro* and suggest that HuR protein has the potential to stabilize *bcl-2* mRNA in HL60 cells as well as in A431 cells.

Previous studies have shown that nucleolin also stabilizes *bcl-2* transcripts in cell-free decay assays (23). Gel-shift assays presented above provide evidence that HuR and nucleolin can bind to the *bcl-2* 3′-UTR concurrently, suggesting that the two proteins may be present in the cell in common *bcl-2* mRNP complexes. This raises the question of whether the functional activities of the two proteins are overlapping or additive or, possibly, synergistic. As a first step to examine this question, the decay of transcripts was examined in the presence of the individual proteins or both HuR and nucleolin. In these assays, low concentrations of the recombinant proteins were added to decay reactions to produce partial protection of the transcripts, to be able to detect additive or synergistic effects. As shown in Fig. 8, addition of 10 nmol/L nucleolin to the decay assay increased the half-life of *bcl-2* transcripts from 19 to 22 minutes, whereas the half-life of transcripts in the presence of 10 nmol/L HuR was 24 minutes. Interestingly, addition of 10 nmol/L of both nucleolin and HuR resulted in a much greater effect on RNA decay, increasing the half-life to 54 minutes.

To assess whether the combined effect of HuR and nucleolin on the rate of RNA decay was additive or synergistic, we used

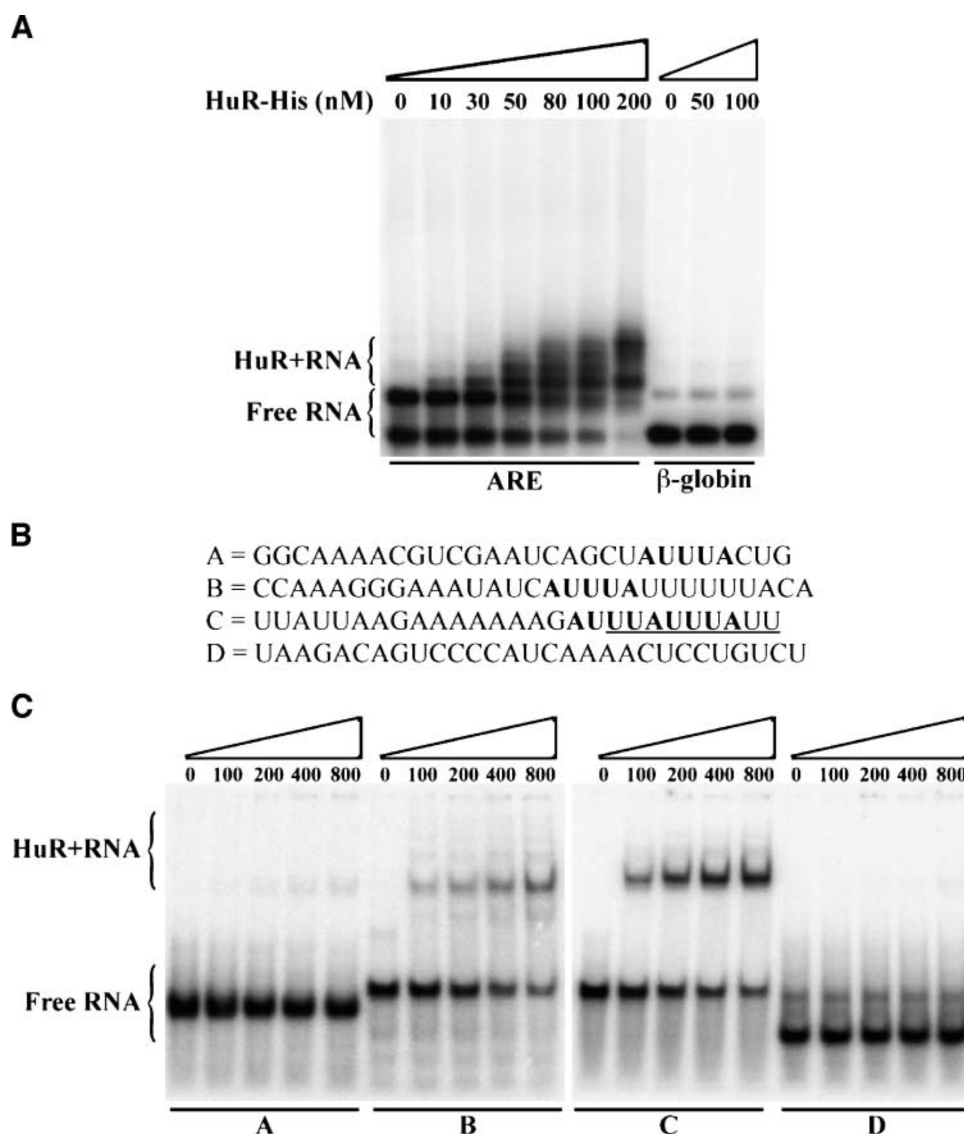

FIGURE 3. RNA binding activity of HuR-His. **A.** ^{32}P-ARE$^{bcl\text{-}2}$ RNA (1.2 nmol/L) was incubated in buffer alone or with purified recombinant HuR (HuR-His; 10-200 nmol/L, as indicated). Samples were separated on a 1.5% agarose gel, which was dried and analyzed by phosphorimaging. As a negative control, *β-globin* RNA was incubated with different concentrations of HuR-His (0-100 nmol/L, as indicated). **B.** Sequence of fragments of ARE$^{bcl\text{-}2}$ used for binding studies in **C**. Fragments A to D are contiguous in ARE$^{bcl\text{-}2}$. **C.** ^{32}P-ARE fragments (A to D, at 25 nmol/L each) were incubated in buffer alone or with purified recombinant HuR (HuR-His; 100, 200, 400 or 800 nmol/L). Samples were separated on a native 8% polyacrylamide gel, which was dried and analyzed by phosphorimaging.

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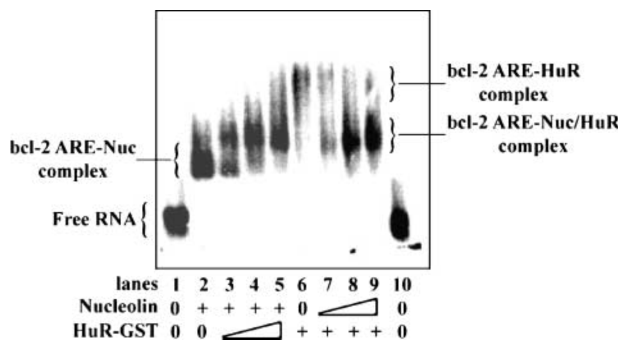

FIGURE 4. Concomitant binding of HuR-GST and ΔNuc-His to ARE^{bcl-2}. ^{32}P-ARE^{bcl-2} RNA (25 nmol/L) was incubated alone or in the presence of 100 nmol/L nucleolin-His (*lanes nucleolin* +) plus 0, 100, 200, and 400 nmol/L HuR-GST (*lanes 2-5*). Similarly, ARE^{bcl-2} was incubated with 100 nmol/L HuR-GST (*lanes HuR* +) plus 0, 100, 200, and 400 nmol/L ΔNuc-His. Samples were analyzed as described in Fig. 3A.

the statistical approach of Chou and Talalay (47), which is based on a single summary index called the "combination index" (see Materials and Methods). In the current context, synergism is characterized by an increase in the time required to achieve a specified percent of RNA reduction comparing the combined to individual effects (see Supplementary Table S1). A combination index between 0.9 and 1.1 indicates additivity, whereas combination index >1.1 indicates synergism (48). The combination indices calculated at each of the three time points of the decay assay (3.5-5.8; Supplementary Table S1) indicate the presence of strong synergism at each time point; that is, in the presence of both HuR and nucleolin, the rate of RNA degradation is strongly reduced relative to that expected under additivity. Using a Bayesian approach to evaluate the statistical significance of the combination index estimates, exceedence probabilities of >0.99 were obtained (Supplementary Table S1), providing strong evidence in support of HuR and nucleolin synergism.

Knockdown of HuR Alters the Polysome Distribution of bcl-2 *mRNA*

In addition to its role in mRNA stabilization, HuR protein has been found to regulate the translation efficiency of some ARE-containing mRNAs in both a positive (32) and a negative (49) fashion. The shRNA knockdown of HuR (Fig. 5) resulted in a moderate but significant decrease in *bcl-2* mRNA levels and a more pronounced decrease in Bcl-2 protein levels. This raises the question of whether HuR may also play a positive role in the regulation of *bcl-2* mRNA translation in A431 cells. To address this question, the relative distribution of *bcl-2* mRNA between monosomes and polysomes (where mRNAs are being actively translated) was examined in A431 cells expressing HuR shRNA. Cytoplasmic lysates were prepared from HuR shRNA knockdown and control A431 cells (scrambled shRNA) and subjected to sucrose density gradient centrifugation analysis. Absorbance at 254 nm was measured across the fractions to confirm the separation of ribosomal subunits, ribosomes, and polysomes (Fig. 9B). As shown in Fig. 9A, the distribution of *bcl-2* mRNA in gradient fractions showed a significant shift to low molecular weight fractions in HuR knockdown A431 cells as compared with cells expressing scrambled shRNA. These results indicate that HuR knockdown produces a redistribution of *bcl-2* mRNA from polysomes (gradient bottom) to pre-polysomal fractions (top), which would lead to decreased rates of translation. *GAPDH* and *tubulin* mRNAs were found to be associated with high molecular weight fractions in HuR knockdown cells (data not shown), suggesting that the observed HuR knockdown effects are specific to *bcl-2* mRNA. Thus, these results provide evidence that HuR regulates *bcl-2* mRNA translation in a positive fashion.

Discussion

Bcl-2 protein is highly expressed in HL60 cells through a process that is thought to involve stabilization of *bcl-2* mRNA. Considerable evidence indicates that stabilization occurs through the interaction of *trans*-acting factors with an ARE element in the *bcl-2* mRNA 3′-UTR. In this study, we addressed the question of whether HuR, which is known to regulate a number mRNAs containing class II ARE motifs, also plays a role in promoting overexpression of *bcl-2* in HL60 leukemia cells.

Earlier studies from our laboratory showed that multiple cytoplasmic factors bind to *bcl-2* mRNA in HL60 cells, including nucleolin (23, 24). Here we have found that HuR coimmunoprecipitates with nucleolin in HL60 cytoplasmic extracts (Fig. 1). The finding that this coprecipitation is RNA dependent suggests that HuR and nucleolin are present in common mRNP. Interestingly, HuR is present in *bcl-2* RNA/protein complexes formed in HL60 both *in vitro* (Fig. 2A) and *in vivo* (Fig. 2B). Moreover, in RNA binding assays with recombinant HuR and nucleolin, RNA complexes containing both proteins were observed (Fig. 4). Concurrent binding of the two proteins to ARE^{bcl-2} RNA *in vitro* is consistent with the concept that HuR and nucleolin are components of *bcl-2* mRNP complexes in HL60 cells.

To define the HuR binding site on the ARE^{bcl-2}, binding of HuR to fragments of the ARE^{bcl-2} was analyzed (Fig. 3B and C). Efficient binding of HuR was only detected with fragments B and C. Fragment B contains one AUUUA pentamer and is U-rich, which may be the determinants for HuR binding (26, 50). Fragment C contains the nonamer UUAUUUAUU sequence, which Zubiaga et al. (51) found to be a minimal sequence for HuR-mediated RNA destabilization. This fragment also contains tandem AUUUA pentamers, which alone are not sufficient for HuR binding but may contribute to binding to larger fragments (26). Given that HuR binds to fragments B and C with much lower affinities than to the intact ARE, it is likely that the recognition site for HuR in *bcl-2* mRNA involves sequences on both fragments and possibly secondary structures formed in the intact ARE (52).

In vitro mRNA decay assays showed that exosome-mediated decay of ARE^{bcl-2} RNA transcripts in HL60 cytoplasmic extracts was reduced in the presence of exogenous HuR protein. Previous studies have shown that nucleolin stabilizes *bcl-2* mRNA in HL60 cells (23) and in chronic lymphocytic leukemia cells (8). Interestingly, assays with both proteins showed that HuR and nucleolin interact synergistically to protect *bcl-2* mRNA from exosome-mediated degradation. It is possible

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that binding of one of the two proteins can induce changes in the secondary structure of the *bcl-2* mRNA, enhancing the binding affinity of the second protein for the mRNA molecule. In fact, induced changes in RNA structure have been frequently described in protein/RNA complexes (53, 54). Alternatively, each protein could bind independently to *bcl-2* mRNA. However, on RNA binding, one or both of the proteins could undergo conformational changes that would favor interactions with the other (i.e., protein-protein interactions that do not occur in the absence of RNA), consequently inducing the formation of an RNA loop. Loop formation would likely have a protective effect against exosomal degradation. Currently, our data do not support one hypothesis over the other.

The demonstration that both HuR and nucleolin stabilize *bcl-2* mRNA indicates that regulation of *bcl-2* mRNA stability *in vivo* is a complex process that can vary, depending, in a cell-specific manner, on the cytoplasmic levels of both nucleolin and HuR. Nucleolin and HuR are predominantly nuclear proteins that shuttle between the nucleus and the cytoplasm. Our findings suggest that aberrant *bcl-2* mRNA stabilization (e.g., in cancer cells) could arise from elevations in the cytoplasmic levels of either nucleolin or HuR or both. In HL60 cells, where Bcl-2 protein expression is very high (7), both HuR and nucleolin seem to play a role in Bcl-2 up-regulation. Although nucleolin (55, 56) and HuR (57) regulate the stability of other mRNAs, *bcl-2* is the only mRNA known, to date, for which both these factors seem to contribute in the regulation of expression. Thus, in HL60 cells, nucleolin and HuR apparently cooperate in promoting cancer cell survival by up-regulating *bcl-2* expression.

Although HuR has been shown to regulate the stability of many target mRNAs (57), there are a few mRNAs for which HuR has been found to influence translation efficiency rather than mRNA stability. For example, *p53* expression is enhanced in RKO cells following UVC irradiation through HuR-mediated increased translation (32). Enhanced translation involves interaction of HuR with the 3′-UTR of *p53* mRNA. Interestingly, the levels and stability of *p53* mRNA were not altered following UVC treatment (32). In contrast, HuR also inhibits the translation of some mRNAs including *p27* (49) and *IGF-IR* (58) mRNAs.

In the case of *bcl-2* mRNA, we have found evidence that HuR influences both mRNA stability and translation. Abdelmohsen et al. (36) also observed an effect of HuR on *bcl-2* mRNA levels in HeLa cells. Stabilization of *bcl-2* mRNA by HuR could be linked to its recruitment of the mRNA to active polysomes. That is, stabilization could be a result of sequestration of *bcl-2* mRNA on polysomes, rather than inhibition of ARE-mediated mRNA decay processes per se. The finding of Mazan-Mamczarz et al. (32) that HuR increases translation

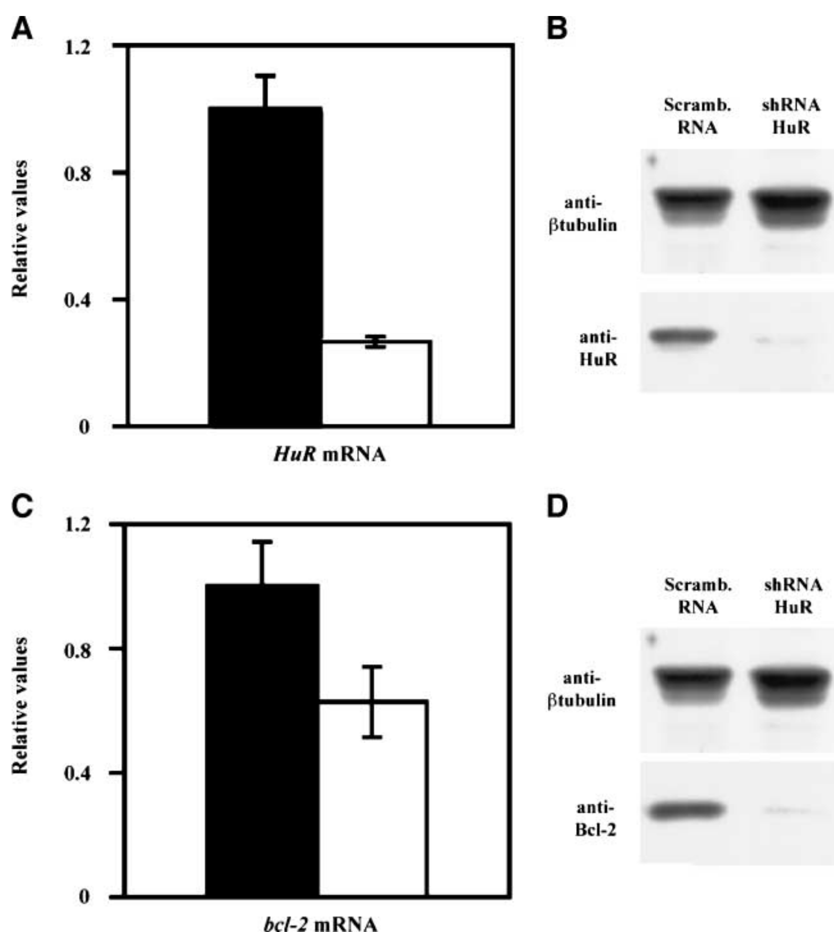

FIGURE 5. The effects of shRNA-mediated knockdown of HuR on *bcl-2* mRNA and protein levels in A431 cells. A431 cells were stably transfected with vectors expressing either an HuR shRNA or a scrambled shRNA. **A.** Whole-cell *HuR* mRNA levels were measured by qRT-PCR. Data are represented relative to the level of *RPL13a* mRNA and are the values from three clones transfected with the same shRNA. Black columns, cells transfected with scrambled shRNA; white columns, HuR shRNA. **B.** Western blot analysis of cytoplasmic extracts from transfected cells probed with anti–β-tubulin or anti-HuR antibodies. **C.** Levels of *bcl-2* mRNA relative to the levels of *RPL13a* mRNA were measured by qRT-PCR. Bar graph is as described in **A** ($P = 0.056$). **D.** Western blot analysis of cytoplasmic extracts prepared from transfected cells probed with anti–Bcl-2 or anti–β-tubulin antibodies.

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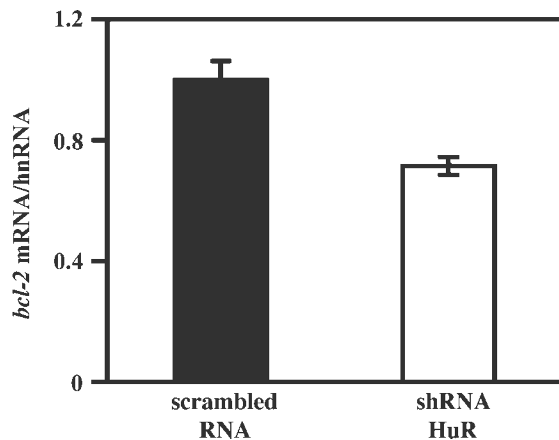

FIGURE 6. The effect of HuR knockdown on nascent and mature *bcl-2* mRNA levels. The ratio of *bcl-2* mRNA and hnRNA levels normalized to *RPL13a* mRNA was measured by RT-PCR in extracts of A431 cells transfected with an HuR shRNA or a scrambled shRNA ($P = 0.03$).

of *p53* mRNA without increasing mRNA stability in RKO cells argues against this notion. Our finding that endogenous HuR binds to ARE$^{bcl\text{-}2}$ RNA in cell extracts and recombinant HuR binds to ARE$^{bcl\text{-}2}$ *in vitro* supports the concept that HuR stabilizes *bcl-2* mRNA in an ARE-mediated manner. Our observation that HuR inhibits *bcl-2* RNA decay in HL60 S100 extracts further supports a direct role of HuR in mRNA stabilization, at least in some cellular contexts. Thus, collectively, our findings support a dual role for HuR in regulating *bcl-2* expression through inhibition of normal mRNA decay processes and translation enhancement.

In mammalian cells, mRNA decay is primarily initiated through shortening of the poly(A) tail (deadenylation; refs. 59, 60). This is followed by 3′-5′ exonucleolytic digestion by the exosome (46, 61), which is a multisubunit complex of exonucleases (62). The presence of an ARE in an mRNA greatly stimulates the rate of exosomal degradation (46). Exosomes do not bind directly to AREs but rather are recruited to ARE-containing mRNAs through interactions with ARE binding proteins (61). Although specific exosome recruiting factors for *bcl-2* mRNA have not been identified yet, the ARE binding protein AUF1 has been found to bind to *bcl-2* mRNA *in vitro* (20). Additionally, Lapucci et al. (20) observed that UVC irradiation of HeLa cells induces apoptosis and down-regulation of *bcl-2* mRNA levels. Decreased *bcl-2* mRNA stability was associated with increased amounts of AUF1/*bcl-2* mRNA complexes following UVC radiation. Thus, AUF1 seems to play a role in *bcl-2* mRNA destabilization during apoptosis. Our finding that HuR protects ARE$^{bcl\text{-}2}$ transcripts from exosome-mediated decay suggests that HuR and AUF1 have opposing roles in regulating the half-life of *bcl-2* mRNA. Thus, the stability of *bcl-2* mRNA may be controlled by the relative abundance of HuR, nucleolin, and AUF1 in the cytoplasm. Ultimately, the levels of Bcl-2 protein will be determined by the stability of *bcl-2* mRNA and the translation efficiency of *bcl-2* mRNA, which are both influenced by HuR.

A particular significance of these new findings is that the antiapoptotic protein Bcl-2 is overexpressed in up to 50% of human cancers (9). Overexpression of Bcl-2 is thought to promote the development of the cancer phenotype and also is associated with resistance to anticancer drugs in some hematologic cancers (9, 10). Therefore, factors that regulate the expression of Bcl-2 are potential targets that could provide novel way(s) of down-regulating Bcl-2 protein. Specifically, our finding that HuR, as well as nucleolin, is likely a part of the *bcl-2* mRNA stabilization complex in HL60 cells suggests that targeting HuR may be a useful way to down-regulate Bcl-2 expression in malignant cells. Our earlier finding that nucleolin down-regulation is associated with decreased Bcl-2 protein levels in Taxol-treated HL60 cells (23) supports the idea that *bcl-2* mRNA stabilizing factors are potential therapeutic targets for new drugs. This is further supported by the recent observation that the nucleolin-targeting DNA aptamer ASl411 destabilizes *bcl-2* mRNA in MCF7 breast cancer cells (12). Collectively, our observations contribute to a fuller understanding of the mechanisms that regulate *bcl-2* expression in cancer cells, which ultimately will help to design new therapeutic agent(s) against cancers in which *bcl-2* expression plays a role in cell survival.

Materials and Methods

Cell Culture

Human HL60 leukemia cells were grown in RPMI 1640 (Cellgro) supplemented with 10% (v/v) heat inactivated fetal bovine serum (Atlanta Biolabs), 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies, Inc.), at 37°C in 5% CO_2. Human A431 epidermoid carcinoma cells were grown in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), penicillin (10 units/mL), and streptomycin (10 μg/mL).

Preparation of RNA Transcripts

ARE$^{bcl\text{-}2}$ 200 nucleotide transcripts for use in gel-shift assays were synthesized using T7 RNA polymerase from *Spe* I-linearized plasmid pCR4-ARE-1A (18). Transcripts labeled with [^{32}P]UTP were synthesized following the manufacturer's protocol (Maxiscript kit, Ambion). The purity of RNA transcripts was monitored by analysis on 6% polyacrylamide/7 mol/L urea gel.

Preparation of HL60 S10 and S100 Cell Extracts

Preparation of S100 cytoplasmic extracts was done following the protocol from Dignam et al. (63) with some modifications. Briefly, HL60 cells were centrifuged at 100 × *g* for 5 min and cell pellets were washed twice with cold PBS. Pellets were suspended in buffer A [10 mmol/L HEPES (pH 8.0), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.1% protease inhibitor cocktail (Sigma), 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 0.5 mmol/L DTT]. After incubation on ice for 10 min, the cells were lysed with a dounce homogenizor and then centrifuged at 10,000 × *g*. The collected supernatant was centrifuged at 100,000 × *g* for 1 h at 4°C. The S100 supernatant was collected, flash-frozen, and immediately stored in aliquots at −80°C.

For S10 cytoplasmic extracts, HL60 cells were washed twice in ice-cold PBS. The cell pellet was resuspended in hypotonic buffer [20 mmol/L HEPES (pH 8.0), 10 mmol/L KCl, 0.5 mmol/L DTT, 0.1% NP40, 5% glycerol, 0.5 mmol/L PMSF, 0.1% protease inhibitor cocktail, 10 mmol/L benzamide, 20 mmol/L sodium fluoride, and 20 mmol/L β-glycerophosphate]

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and incubated at 4°C for 15 min. Suspensions were centrifuged at 10,000 × *g* at 4°C for 10 min. Protein concentration in the extracts was determined by Bradford assay.

Expression and Purification of Recombinant HuR

Plasmid pGEX-HuR (25) containing a recombinant *GST-HuR* gene was a gift from Dr. Henry Furneaux (University of Connecticut Health Center, Farmington, CT). *E. coli* BL21 (DE3) cells transformed with pGEX-HuR were induced with isopropyl-L-thio-B-D-galactopyranoside. Following induction, recombinant HuR protein was purified using the method described previously (18). The purity of the protein was assessed by SDS-PAGE and protein concentration was measured by Bradford assay using BSA as a standard.

To produce HuR-His, the HuR cDNA was subcloned from pGEX-HuR into the pET21a vector (Novagen). Expression and purification were carried out as recommended by the vector manufacturer. Briefly, after protein expression, bacterial cells were lysed in resuspension buffer [20 mmol/L Tris-HCl (pH 8.0), 0.5 mol/L NaCl, 2 mmol/L β-mercaptoethanol, 0.5 mmol/L PMSF] and the supernatant was loaded onto a TALON-NX metal resin (Clontech), pre-equilibrated with resuspension buffer. HuR protein was eluted from the column with resuspension buffer supplemented with 150 mmol/L imidazole. The purity of the protein was monitored by SDS-PAGE. Protein concentration was determined by Bradford assay.

Expression and Purification of Recombinant Nucleolin

For the gel-shift assays, recombinant nucleolin containing residues 284-707 and six histidines (ΔNuc) was purified using a recombinant pET21a plasmid (pET-Nuc-His) carrying a truncated nucleolin gene (64), which was a gift from Dr. France

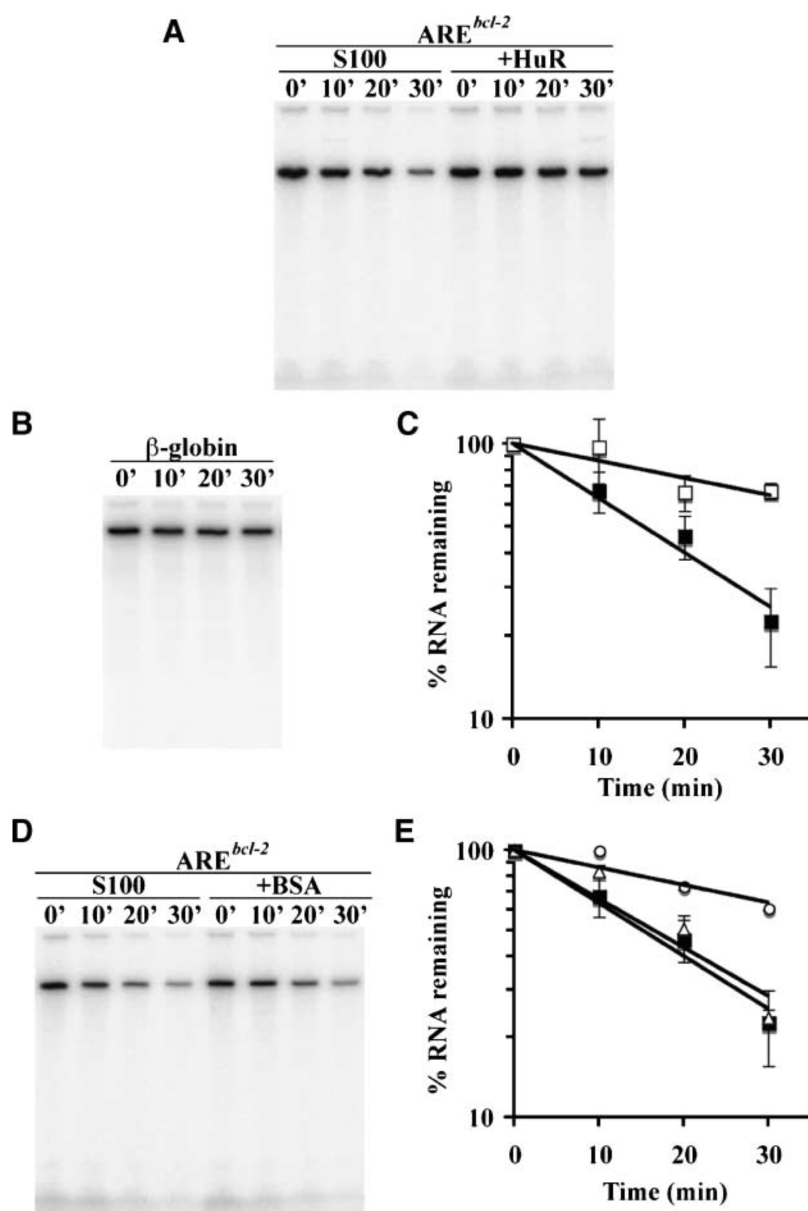

FIGURE 7. Effect of recombinant HuR on decay of *bcl*-2 ARE transcripts in HL60 extracts. 5′-capped ^{32}P-ARE^{bcl-2} and *β-globin* transcripts were incubated in S100 HL60 cell extracts and aliquots were removed at the times indicated. Recovery of RNA was assessed by electrophoresis on denaturing polyacrylamide gels, which were analyzed by phosphorimaging. **A.** Representative assay in which ARE^{bcl-2} transcripts were incubated in unsupplemented S100 extracts or in extracts supplemented with HuR (75 nmol/L). **B.** Representative assay in which *β-globin* transcripts were incubated in unsupplemented S100 extracts. **C.** Semi-log plot of the fraction of RNA remaining versus time of incubation in cell extracts. □, *β-globin* transcript; ■, ARE^{bcl-2}. Both transcripts were incubated in unsupplemented S100 extracts. Points, average of two to six experiments. **D.** Representative assay in which ARE^{bcl-2} transcripts were incubated in unsupplemented S100 extracts or in extracts supplemented with BSA (75 nmol/L). **E.** Semi-log plot of the fraction of RNA remaining versus time of incubation in cell extracts. ■, S100 extracts; ○, S100 supplemented with HuR-His (75 nmol/L); △, S100 supplemented with BSA (75 nmol/L). Points, average of two to six experiments. Error bars for open circles are smaller than the symbol.

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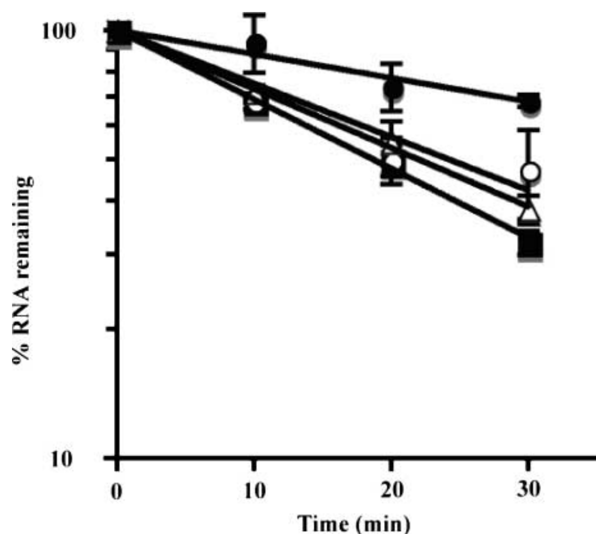

FIGURE 8. Combined effect of HuR and nucleolin on ARE^{bcl-2} transcript decay. Decay assays were done as described in Fig. 7, except that the concentrations of recombinant proteins were reduced to 10 nmol/L. ^{32}P-ARE^{bcl-2} transcripts were incubated with unsupplemented S100 extracts (■). S100 extracts were supplemented with HuR-His (○), with nucleolin-His (△), and with both 10 nmol/L HuR-His and 10 nmol/L nucleolin-His (●).

Carrier (University of Maryland, Baltimore, MD). For the decay assays, recombinant nucleolin with NH_2-terminal and COOH-terminal deletions (ΔNucΔRGG) was purified from a pET21a plasmid encoding nucleolin residues 284-643 and 6 His. Purification of recombinant nucleolin proteins was done as described previously (23). Protein purity was assessed by SDS-PAGE and protein concentration was measured by Bradford assay.

HuR and Nucleolin Coimmunoprecipitation

HL60 S10 cytoplasmic extracts were precleared with protein A/G-agarose beads (Santa Cruz) in coimmunoprecipitation buffer [20 mmol/L HEPES (pH 8.0), 150 mmol/L NaCl, 0.5 mmol/L DTT, 5 mmol/L EDTA, 0.05% NP40, 5% glycerol, 0.5 mmol/L PMSF, 0.1% protease inhibitor cocktail, 20 mmol/L sodium fluoride, 20 mmol/L β-glycerophosphate, 10 mmol/L benzamide]. The suspension was centrifuged and the supernatant incubated with either RNase inhibitor or RNase I. After incubation for 30 min at 37°C, protein A/G-agarose beads preincubated with anti-nucleolin or IgG (Santa Cruz) were added, and incubation proceeded for 16 h at 4°C. Beads were washed with coimmunoprecipitation buffer and resuspended in SDS-loading buffer. Proteins were subjected to SDS-PAGE, and Western blot analysis was done with anti-HuR, anti-nucleolin (Santa Cruz), or anti-GAPDH (Chemicon) antibodies.

RNA Gel Mobility Shift Assays and Antibody Supershift Assay

^{32}P-ARE RNA transcripts were mixed with 3 μg of HL60 S10 cytoplasmic extracts in RNA binding buffer [10 mmol/L Tris-HCl (pH 8.0), 50 mmol/L KCl, 2 mmol/L DTT, 0.5 mmol/L EDTA, 0.25 mg/mL BSA, and 0.25 mg/mL tRNA]. Monoclonal anti-HuR antibody (0.4 μg), monoclonal anti-nucleolin antibody (0.4 μg), or IgG antibody (0.4 μg; Santa Cruz Biotechnology) was added. Samples were incubated for 5 min at 30°C, followed by 5 min on ice, except when noted otherwise. Reaction products were separated by electrophoresis on a 1.2% agarose gel. The gel was dried on Nytran SPC membrane (Whatman) and analyzed by phosphorimaging using a STORM PhosphorImager and Image Quant software (GE).

To assay for HuR-His binding to ARE^{bcl-2}, purified recombinant HuR-His protein (10-200 nmol/L) was incubated with ^{32}P-ARE^{bcl-2} RNA transcripts in RNA binding buffer. Samples were incubated for 5 min at 30°C, followed by 5 min on ice, then separated on a 1.5% agarose gel. As a negative control, ^{32}P-β-globin RNA (23) was incubated with HuR-His as described for ARE^{bcl-2}.

Oligoribonucleotides corresponding to contiguous fragments within the ARE^{bcl-2} sequence (NM_000633: nucleotides 1392-1503) were chemically synthesized (Dharmacon). The sequences of the RNA fragments were as follows: fragment A, GGCAAAACGUCGAAUCAGCUAUUUACUG; fragment B, CCAAAGGGAAAUAUCAUUUAUUUUUUACA; fragment C, UUAUUAAGAAAAAAAGAUUUAUUUAUU; fragment D, UAAGACAGUCCCCAUCAAAACUCCUGUCU. Fifty nanograms of each fragment were kinased using [γ-^{32}P]ATP and T4 polynucleotide kinase (Fisher). Mobility shift assays were done as described above. Briefly, purified recombinant HuR-His protein (100-800 nmol/L) was incubated with ^{32}P-labeled fragments in RNA binding buffer for 5 min at 30°C, followed by 5 min on ice. Samples were then analyzed on an 8% polyacrylamide gel.

RNA Immunoprecipitation Assay

Immunoprecipitation of protein/RNA complexes was done as described by Niranjanakumari et al. (38). Briefly, HL60 cells were cross-linked with 1% formaldehyde (Sigma). Cross-linking reactions were quenched by the addition of glycine (pH 7.0) at 0.25 mol/L final concentration. The cells were washed with ice-cold PBS, resuspended in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 8.0), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mmol/L EDTA, 150 mmol/L NaCl] containing phosphatase and protease inhibitors (Sigma), and lysed by sonication. The extract was centrifuged at 12,000 × *g* for 10 min at 4°C. Aliquots of cell lysate (1 mg of total protein) were precleared with protein A/G-agarose beads (Santa Cruz). Equal aliquots of the precleared supernatant were incubated with protein A/G-agarose beads that had been preincubated with IgG, monoclonal anti-HuR (Santa Cruz), or monoclonal anti-CD55 (Ancell) antibody. The agarose beads were washed with high-stringency radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 8.0), 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA, 1 mol/L NaCl, 1 mol/L urea, 0.2 mmol/L PMSF] and then recovered by centrifugation. Resuspended beads were incubated at 70°C for 45 min to reverse the cross-links. Nucleic acids were extracted with TRI reagent (Sigma) and treated with RNase-free DNase (Ambion). The purified RNA was used as a template to synthesize cDNA using random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Fisher) according to the manufacturer's protocol. Real-time PCR was done with

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SYBR Green Mix (Quanta Biosciences) using primers specific for *bcl-2* mRNA (reverse, 5′-TGCAATCCACTGTCACTCTTGC-3′; forward, 5′-CTGCGTAAATCCATGCACCTAAACC-3′) and for *GAPDH* mRNA (reverse, 5′-GGCATGGACTGTGGTCATGAG-3′; forward, TGCACCACCAACTGCTTAGC-3′).

HuR Knockdown in A431 Cells

A shRNA expressing vector was constructed by inserting synthetic double-stranded oligonucleotides between the *Bam* HI and *Hin*dIII sites of the polymerase III gene promoter on vector pSilencer (Ambion). The shRNA sequence was targeted to nucleotides at position 377-397 of HuR cDNA (AACACGCTGAACGGCTTGAGG; GenBank accession no. BC0003376). A431 cells were transfected with this vector using Lipofectamine reagent (Invitrogen). As a negative control, cells were transfected with a pSilencer vector expressing a scrambled hairpin shRNA of the same length that does not match any known human gene. G418-resistant clones were collected and screened by Western blot analysis. Clones that showed significant reduction in HuR protein levels were used for measurement of *HuR* and *bcl*-2 mRNA levels by qRT-PCR. The GeNorm program (65) was used to select ribosomal protein 13a (*RPL13a*) as the optimal reference housekeeping gene for the qRT-PCR assays. Primers for *HuR* and *bcl-2* mRNAs were purchased from Qiagen. The sequences of primers for *RPL13a* were 5′-CCTGGAGGAGAGAGGAAAGAGA-3′ (forward) and 5′-TTGAGGACCTCTGTGTATTTGTCAA-3′ (reverse).

In shRNA knockdown cells, Western blot analysis of Bcl-2, β-tubulin, and HuR proteins was done with cytoplasmic extracts. Equal quantities (25 μg) of supernatant protein from HuR knockdown and control cells were separated on an SDS-10% polyacrylamide gel. Monoclonal anti–Bcl-2, anti–β-tubulin, or anti-HuR antibodies (Santa Cruz) were used to probe for the respective proteins.

Nascent and Mature bcl-2 mRNA Levels

Total RNA was purified with TRI reagent (Sigma) from cytoplasmic extracts of control and HuR knockdown A431 cells. The RNA was used as a template to synthesize cDNA as described above. hnRNA and mRNA levels were determined by PCR using primers specific for *bcl-2* mRNA (reverse, 5′-ACAGCCTGCAGCTTTGTTTC-3′; forward, 5′-GAGGATTGTGGCCTTCTTTG-3′) and primers specific for *bcl-2* hnRNA (reverse, 5′-CTGTGGATGACTGAGTACCTGAAC-3′; forward, 5′-AAGCAACAACTCTGATTTTATTTCG-3′); primers for *RPL13a* mRNA were as described above. The PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining. Densitometry of the bands was determined using ImageQuant TL (version 7.0, GE).

In vitro mRNA Decay Assays

To assay for exosome-mediated decay, 5′-capped, ^{32}P-labeled transcripts were prepared from *Spe*I-linearized pCR4-*bcl*-ARE and pCR4-β-globin plasmids (23) using a mMessage mMachine T7 kit (Ambion). RNA transcripts were extracted with TRI reagent (Sigma) and analyzed for purity on denaturing gels before use. Decay assays were done following the protocol previously described (45, 46). Briefly, HL60 S100 extracts were preincubated with RNaseIn (Promega). ^{32}P-labeled transcripts were mixed with extracts that were unsupplemented or supplemented with purified recombinant HuR-His protein, ΔNucΔRGG, or BSA. Reactions were incubated at 30°C, and aliquots were removed at various times, then added to stop buffer (18), and immediately extracted with TRI reagent. Purified RNA was analyzed on 8% polyacrylamide gels containing 8 mol/L urea. After electrophoresis, gels were dried and analyzed by phosphorimaging. Densitometry of the bands was done using the ImageQuant TL.

To determine if the addition of nucleolin and HuR to extracts resulted in additive or synergistic effects on RNA decay, the approach of Chou and Talalay (47) was used, in which a combination index was calculated for each time point. The combination index is a sum of individual dose ratios (dose = time), where each ratio compares the doses required to achieve

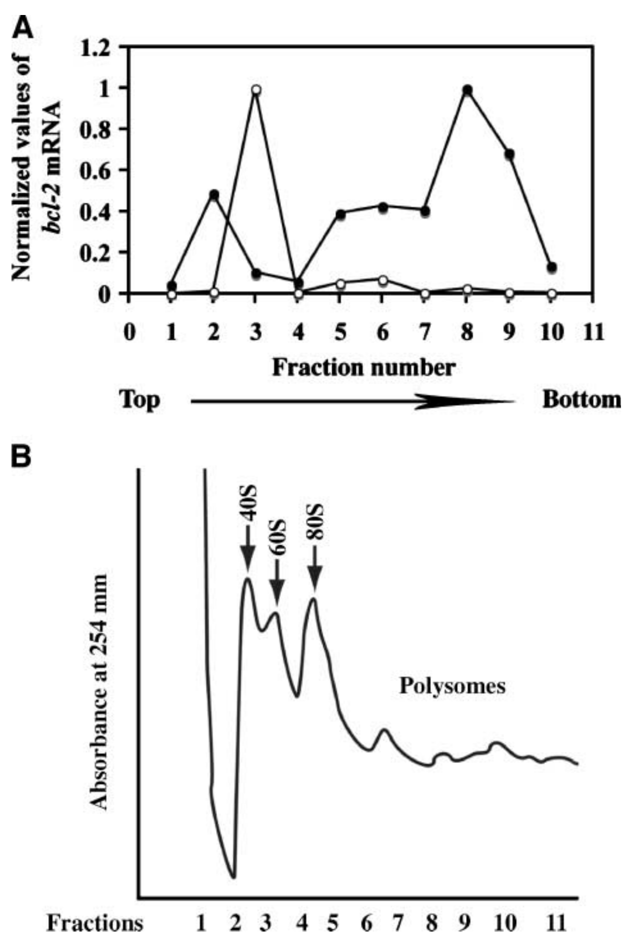

FIGURE 9. Effect of HuR knockdown on distribution of *bcl-2* mRNA in sucrose density gradients. Cytosolic extracts from A431 cells transfected with HuR shRNA (○) or scrambled RNA (●) were applied to a 7% to 47% sucrose density gradient. After centrifugation, (**A**) fractions were collected and *bcl-2* mRNA levels in the fractions were determined by qRT-PCR. mRNA values were normalized by setting the value of the peak fraction at 1.0. Similar results were obtained in a second independent experiment; (**B**) absorbance was monitored continuously at 254 nm using an ISCO-UA5 (model 640) density gradient fraction collector. A similar profile was observed with gradients containing extracts from A431 cells transfected with scrambled RNA.

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a specified effect in the combined and individual settings. In the current context, synergism is characterized by an increase in the time required to achieve a specified percent RNA reduction comparing the combined to the individual effects.

Polysome Profile Analysis from HuR Knockdown A431 Cells

Preparation of cytoplasmic extracts and polysome profile analysis was carried out as described in detail previously (66). After ultracentrifugation, the tubes were punctured at the bottom and fractions were displaced upward using Fluorinert FC-40 (Sigma). Absorbance was monitored continuously at 254 nm using an ISCO-UA5 (model 640) density gradient fraction collector. Ten fractions were collected and total RNA was extracted from each using TRI reagent. The distribution of *bcl-2* mRNA in each fraction was determined by qRT-PCR as above.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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