Hu Antigen R (HuR) Functions as an Alternative Pre-mRNA Splicing Regulator of Fas Apoptosis-promoting Receptor on Exon Definition*

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Exclusion of exon 6 by alternative RNA splicing of the primary transcript of the apoptosis receptor Fas produces a soluble isoform that prevents programmed cell death. I report that anti-apoptotic regulator Hu antigen R (HuR, ELAVL1), a member of the embryonic lethal, abnormal vision, Drosophila-like (ELAVL) family, promotes Fas exon 6 skipping by binding to an exonic splicing silencer. HuR inhibits the association of U2 small nuclear ribonucleoprotein (snRNP) auxiliary factor 65 kDa (U2AF65) with the upstream 3’ splice site, without decreasing recognition of the downstream 5’ splice site by U1 snRNP but by antagonizing the role of TIA-1 (T-cell intracellular antigen 1)/TIAR (TIA-1 related protein) on exon definition. Remarkably, U1 snRNP-mediated recognition of the 5’ splice site is partially required for efficient U2AF65 inhibition. Further, the silencing capacity of HuR as splicing regulator resides in the RRM1 and hinge-RRM3 domains. Taken together, these results support a functional link between HuR as repressor of alternative Fas splicing and the molecular mechanisms modulating programmed cell death.

The molecular basis of the regulation of alternative splicing is linked to splice site selection. Thus, authentic splicing sequence elements are often indistinguishable from cryptic sequences hidden in human pre-mRNAs, raising the question of how the spliceosomal machinery can define an authentic splicing motif. Splice site selection depends on balancing of positive and negative sequence elements and on changes in the activity and/or amount of cellular splicing factors under physiological or pathological conditions (1). Although alternative splicing is widespread in human biology (2), few factors involved in regulation of alternative splicing of human mRNAs have been identified, and even fewer have been characterized (3).

T-cell intracellular antigen 1 (TIA-1)2 and TIA-1 related protein (TIAR) are both involved in controlling splice site choice. They were first identified as apoptosis-promoting factors (4). TIA-1 and TIAR contain three RNA recognition motifs (RRMs) and a guanine-rich C-terminal domain (5). Both proteins regulate the alternative splicing of various human and Drosophila pre-mRNAs (FGFR-2 (fibroblast growth factor receptor-2), msl-2, TIA1, CTRF (cystic fibrosis transmembrane conductance regulator), Fas, and COL2A1 (type II procollagen gene) (6–11)) through binding to U-rich stretches, facilitating atypical 5’ splice site recognition by U1 snRNP. TIAR can also enhance U6 snRNP assembly on a pseudo-5’ splice site followed by uridines in the calcitonin/calcitonin gene-related peptide pre-mRNA (12).

The RNA-binding proteins HuA (HuR, ELAVL1), HuB (Hel-N1, ELAVL2), HuC (ELAVL3), and HuD (ELAVL4) comprise the embryonic lethal, abnormal vision, Drosophila-like (ELAVL) family (13). Expression of HuB, HuC, and HuD proteins is limited to neuronal tissues/cells, whereas HuR is ubiquitously expressed (14). HuB, HuC, and HuD proteins have been recently identified as splicing regulators in neuron-like cells (15). The primary structures of the Hu proteins are highly conserved (70%) and contain three RNA recognition motifs (RRMs). The region between RRM2 and 3 defines a basic hinge domain containing a nuclear localization signal, which allows Hu proteins to shuttle between the cytoplasm and the nucleus (16). HuR has been shown to modulate a variety of post-transcriptional decisions (17, 18) in several key cellular processes, including immune response (19), exposure to stress agents (20, 21), cell survival (22, 23), inflammation (24), cell cycle control (25), differentiation program (26), oncogenesis (27), and replicative senescence (28).

Human gene functions can change by producing multiple functional isoforms, even antagonistic, from a single gene in different tissues, developmental stages, or even in pathological situations (1). One example is the human Fas (CD95/APO-1) pre-mRNA, which encodes a type 1 transmembrane protein that mediates apoptosis on Fas ligand binding (29). Fas is a death receptor that is constitutively expressed by most tissues (29). Alternative splicing of Fas pre-mRNA can regulate the sensitivity of Fas-expressing cells to Fas-induced apoptosis.

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2 The abbreviations used are: TIA-1, T-cell intracellular antigen-1; TIAR, TIA-1 related protein; ELAVL, embryonic lethal, abnormal vision Drosophila-like family; GFP, green fluorescent protein; HuR, Hu antigen R; MBP, maltose-binding protein; PTB, polypyrimidine tract-binding protein; RRM, RNA recognition motif; siRNA, small interference RNA; snRNP, small nuclear ribonucleoprotein; snRNA, small nuclear RNA; U2AF65, U2-snRNP auxiliary factor 65 kDa; URE6, uridine-rich sequence located in Fas exon 6; RT-PCR, reverse transcription-PCR; WT, wild type; ARE, AU-rich sequence element; hnRNP, heterogeneous nuclear ribonucleoprotein.
The protein isoform of the mRNA lacking exon 6 encodes the soluble form of the receptor able to inhibit Fas signaling (30). This process is regulated in a number of physiological situations, including activation-mediated T-cell death, and failure to switch between isoforms underlies autoimmune lymphoproliferative disorders (31).

Despite extensive characterization and the predominant nuclear localization of HuR, the idea that this protein functions as splicing regulator remains unclear because no target sequences of HuR have been identified. I show here that HuR is a novel regulator of alternative Fas splicing.

**EXPERIMENTAL PROCEDURES**

**Constructs and Recombinant Protein Preparations**—All the constructs used have been previously described (10, 32, 33). Recombinant MBP, MBP-HuR, His-PTB, GST-TIA-1b, or GST-TIARb fusion proteins were expressed in *Escherichia coli* and purified as described (10, 32, 33).

**Cell Cultures, DNA Transfection, and RNA Interference Analysis**—HeLa cells were grown and transfected with 20 nm (final concentration) of either a control siRNA (non-silencing off-target siRNA effects, I used an siRNA-resistant variant of gene harboring a mutation at the polypyrimidine tract of intron 5 (Fas U-20C) that increases exon 6 skipping (10). To exclude double transfection, siRNAs were transfected first followed by transfection of plasmid DNA 72 h later. The cells were collected after 24 h.

**Protein Analysis, RNA Isolation, and RT-PCR Analysis**—Immunoblotting were carried out using the following antibodies: anti-U2AF65 (MC3, kindly provided by Dr. J. Valcárcel), anti-TIA-1 (C-20), anti-TIAR (C-18), anti-hnRNPC1/C2, and anti-HuR (3A2) from Santa Cruz Biotechnology; anti-α-tubulin (B-5-1-2, Sigma); and anti-GFP (IL-8, Clontech). Cytoplasmic RNAs were purified and analyzed as described previously (7, 10).

**Fluorescence Microscopy**—HeLa cells were grown for 24 h on coverslips and processed as described previously (34). Endogenous HuR/TIA-1/TIAR antibody complexes, To-pro-3, or GFP-fused proteins were viewed by confocal microscopy.

**In Vitro Splicing, RNA-RNA, and RNA-Protein Interaction Assays**—In vitro transcription and splicing assays were carried out as described previously (7) using capped RNAs synthesized by T7 transcription from PCR templates. UV cross-linking and immunoprecipitation, psoralen-mediated cross-linking, and RNase H-mediated U1 inactivation were performed as described (10).

**RESULTS**

**HuR siRNA-mediated Depletion Promotes Fas Exon 6 Inclusion**—HuR is an RNA-binding protein mainly localized in the nucleus of HeLa cells (>90%), with nucleolar exclusion, whereas TIA-1 and TIAR showed a characteristic nuclear-cytoplasmic localization pattern (Fig. 1, A–C). However, both endogenous and ectopic HuR and TIA-1 proteins showed similar nuclear colocalization (Fig. 1, A–C), suggesting that they might have related functions in nuclear events such as, for example, the control of alternative pre-mRNA splicing. To test this idea, I reduced the abundance of endogenous HuR in HeLa cells (80–90%) by RNA interference, resulting in increased levels of Fas exon 6 inclusion (Fig. 2, A and B, compare lanes 2–4 with lanes 1–3, respectively). In these experiments, to detect reliable differences in exon 6 inclusion, I have used a Fas minigene harboring a mutation at the polypyrimidine tract of intron 5 (Fas U-20C) that increases exon 6 skipping (10). To exclude off-target siRNA effects, I used an siRNA-resistant variant of HuR, refractory to the siRNA directed to the 3′-untranslated region of HuR, in line with previous data (33). The results indicate that the resistant protein can provide the full range of their
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An Exonic Splicing Silencer Mediating HuR Repression—Cotransfection of Fas-derived minigenes (10, 34) and plasmids expressing either GFP or GFP-HuR in HeLa cells was used to characterize the sequence through HuR function as a Fas splicing repressor. HuR overexpression over wild type (WT) Fas minigene promoted exon 6 skipping (Fig. 2, C and D, compare lane 1 with lane 4), indicating that HuR down-regulates exon 6 inclusion. Nevertheless, the mutant m0 minigene, with replacement of a uridine-rich sequence on exon 6 (URE6), was fully insensitive to equivalent levels of HuR overexpression (Fig. 2, C and D, compare lane 2 with lane 5). In these studies, TIAR overexpression and the effects on U-20C and mURI6 reporter minigenes were used as positive and negative controls of exon 6 inclusion and skipping, respectively (Fig. 2, C and D, lanes 3 and 6–9). I conclude that HuR recruitment to the URE6 sequence induces Fas exon 6 skipping.

To further document HuR function through the URE6 element, this sequence was substituted for two MS2 stem-loop binding sites in tandem for the bacteriophage MS2 protein (Fas 2xMS2 minigene) (10). Cotransfection with a plasmid expressing an MS2-HuR fusion protein led to significant exon 6 skipping (Fig. 2E, lane 4), whereas expression of GFP or MS2 proteins did not (Fig. 2E, lanes 1 and 2). As I described previously (10), expression of an MS2-PTB fusion protein also led to efficient exon 6 skipping (Fig. 2E, lane 3). However, the URE6 substitution rendered the minigene insensitive to PTB or HuR overexpression (Fig. 2E, lanes 5 and 6). The simplest interpretation of all these results is that URE6 sequence silences exon 6 inclusion in a HuR-dependent way.

Fas Splicing Regulation in Vivo and in Vitro by HuR and TIA-1/TIAR Antagonism—Given that antagonism between TIA-1 and HuB/HuC/HuD proteins has been reported previously (15), in vivo splicing experiments in HeLa cells were performed by cotransfection of WT or m0 Fas minigenes and plasmids expressing GFP, HuR alone, or HuR with TIA-1 or TIAR activity even under silencing conditions (Fig. 2A and B, compare lanes 2, 4, and 5 with lanes 1, 3, and 4, respectively), thus strongly arguing that HuR does promote exon 6 skipping. These results suggest that HuR might function as a Fas exon 6 splicing repressor.

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mulation (Fig. 3C, lanes 6 and 7). Most of the effects observed previously (Figs. 2, D and E, and 3B) were detectable and recapitulated with the m0 mutant containing mutation of URE6 sequence. For example, this mutant remained insensitive to HuR excess (Fig. 3C, compare lanes 2 and 9 with lane 10). Similarly, the addition of purified recombinant PTB reduced the 5–6 spliced product (Fig. 3C, lane 12), whereas the m0 mutant was insensitive to PTB excess (Fig. 3C, lane 13). Overall, the results recapitulate the effects of cis-acting sequences and trans-acting factors observed ex vivo and indicate that HuR acts directly through the exonic silencer URE6.

Interaction between HuR and URE6—To confirm that HuR binds to the exonic silencer URE6, RNA binding assays were done. Cross-linking assays with an RNA corresponding to Fas exon 6 (E6 WT) indicated that the addition of purified recombinant HuR supplemented with HeLa nuclear extracts results in a significant increase in HuR cross-linking, without affecting the overall pattern of cross-linking of other proteins (Fig. 4A, compare lanes 2 and 3), whereas mutation of the URE6 sequence (E6 m0) reduced HuR cross-linking 5–10-fold (Fig. 4A, lanes 5 and 6). This preferential binding to the URE6-containing RNA was also observed in UV-cross-linking plus immunoprecipitation assays. In fact, HuR present in HeLa nuclear extracts was cross-linked to the radioactively labeled WT RNA corresponding to the Fas exon 6 followed by HuR immunoprecipitation (Fig. 4B, lane 2), and this cross-linking was significantly reduced by mutation of the URE6 sequence (Fig. 4B, lane 4). These results indicate that HuR binds to the URE6 sequence. Additionally, purified recombinant HuR was incubated with RNAs corresponding to the WT or m0 Fas exon 6 (Fig. 4C). HuR binding was significantly reduced (4–5-fold) by mutation of the URE6 sequence (Fig. 4C, compare lanes 1–6 with lanes 7–12). This result indicates that HuR binds directly to URE6. In line with the function of Hu proteins in other RNAs (35, 36), my results showed cooperative HuR binding to Fas RNA substrates since the fastest migrating HuR-RNA complexes accumulate according to protein concentration, rather than sequentially (Fig. 4C). Taken together, these results suggest that the URE6 sequence can function as a high affinity site facilitating HuR recruitment and favoring multimerization.

Mechanism of Fas Splicing Regulation by HuR—Fas intron 5 splicing is completely dependent on the presence of the 5’ splice site of intron 6 and downstream sequences as well as recognition of these sequences by U1 snRNP and TIA-1/PTB, which facilitate exon definition and splicing of the upstream intron (10). Given that HuR can form multimers and so alter recognition of the sequences at the 5’ splice site in intron 6, I analyzed the effect on psoralen-mediated cross-linking between U1 snRNA and the 5’ splice site region of Fas exon 6 when adding recombinant purified HuR protein to nuclear extracts. As shown in Fig. 4D, excess HuR did not diminish but rather increased interaction between U1 snRNA and 5’ splice site (Fig. 4D, compare lanes 1 and 5 with lanes 4 and 8, respectively). This analysis was repeated with PTB as a positive control (Fig. 4D, compare lanes 1 and 5 with lanes 3 and 7, respectively). However, U1 snRNA binding to the 5’ splice site sequence was clearly reduced in extracts with U1 snRNP inactivated by RNase H-mediated digestion of U1 snRNA 5’ end (Fig. 4D, lanes 2 and 6). I conclude that HuR stabilizes U1 snRNP recruitment to the 5’ splice site of exon 6.

I have previously shown that Fas intron 5 splicing was facilitated by an exon definition phenomenon, probably due to stabilization of U2AF binding mediated by U1 snRNP association with the downstream 5’ splice site across the exon (3, 37). Consistent with this, U2AF65 cross-linking was abolished in the
Finally, I tested whether the residual cross-linking of U2AF65 PTB (Fig. 4A, lane 5) was completely inhibited by HuR (Fig. 4F, lane 7) but not by PTB (Fig. 4F, lane 6; the stronger background is due to the longer exposure required to detect the low levels of U2AF65 cross-linking in the absence of a 5' splice site). Taken together, these results indicate that HuR binding to the URE6 element interferes with U2AF65 association with the 3' splice site region and suggest that HuR inhibition mainly requires the 5' splice site of intron 6 (i.e., interactions across the exon that stabilize U2AF65 binding to the 3' splice site by U1 snRNP) and a direct interaction with the U2AF65 binding site. I conclude that HuR can mediate Fas splicing repression through its capacity to interfere in exon definition.

**RRM1 and Hinge-RRM3 Domains of HuR Are Necessary and Sufficient to Promote Exon 6 Exclusion—**Human HuR protein is made up of two RRMs arranged in tandem near the N terminus followed by a basic hinge domain and a third RRM near the C terminus (14, 38) (Fig. 5A). Structures of peptide-RNA complexes containing RRMs 1 and 2 of HuR protein indicated that these domains are sufficient for specific recognition of AU-rich sequence element (ARE) substrates (36, 39). However, the hinge domain contributes significant binding energy to HuR-ARE complex formation in an ARE length-dependent way, whereas the RRM3 domain is required for cooperative assembly of HuR oligomers on RNA (36). I aimed to establish the domain(s) through HuR functions as a splicing repressor with a tethering approach to test the functionality of each protein domain as fusion protein to MS2. First, I analyzed how overexpression of the RRM1–2 and hinge-RRM3 domains affects the Fas2xMS2 minigene (Fig. 5B). The capacity of both domains to promote exon 6 skipping was similar to full MS2-HuR fusion protein (Fig. 5B, compare lane 2 with lanes 3 and 6). However, on separating and analyzing each domain individually, only RRM1 had sufficient repressor activity to promote exon 6 skipping (Fig. 5B, compare lane 3 with lanes 4 and 5 and

absence of a 5' splice site (Fig. 4E, lane 6) and in the presence of excess HuR (Fig. 4E, lane 4) at levels equivalent to those corresponding to affinity chromatography U2AF65-depleted extract (Fig. 4E, lane 5). U2AF65 cross-linking was also reduced in the presence of PTB (Fig. 4E, lane 3) as well as in extracts in which U1 snRNP was inactivated by RNase H-mediated digestion of U1 snRNA 5' end (Fig. 4E, lane 2). Furthermore, I demonstrated that inhibition of association of U2AF65 with the 3' splice site by HuR occurs in an URE6-dependent manner since this association is re-established with m0 mutant (Fig. 4F, lanes 1–3). Finally, I tested whether the residual cross-linking of U2AF65 observed in the absence of the 5' splice site of intron 6 (not mediated by exon definition) was inhibited by HuR. In the absence of intron 6 sequences, U2AF65 cross-linking did not depend on the integrity of U1 snRNA (Fig. 4F, lane 5). Remarkably, U2AF65 cross-linking was completely inhibited by HuR (Fig. 4F, lane 7) but not by PTB (Fig. 4F, lane 6; the stronger background is due to the longer exposure required to detect the low levels of U2AF65 cross-linking in the absence of a 5' splice site). Taken together, these results indicate that HuR binding to the URE6 element interferes with U2AF65 association with the 3' splice site region and suggest that HuR inhibition mainly requires the 5' splice site of intron 6 (i.e., interactions across the exon that stabilize U2AF65 binding to the 3' splice site by U1 snRNP) and a direct interaction with the U2AF65 binding site. I conclude that HuR can mediate Fas splicing repression through its capacity to interfere in exon definition.

**FIGURE 4.** HuR inhibits U2AF65 binding by interfering with the molecular interactions that lead to exon exclusion. A and B, HuR cross-links to Fas exon 6 URE6 sequence. A, RNAs uniformly labeled with 32P-uridine corresponding to WT or mutated (m0) Fas exon 6 were incubated with none (lanes 1 and 4) or HeLa nuclear extracts (NE) in the presence of either MBP (lanes 2 and 5) or MBP-HuR (lanes 3 and 6), irradiated with short wave UV light, treated with RNase A, and followed by immunoprecipitation with anti-hnRNPC1/C2 (used as a control) or anti-HuR antibodies. The RNA-protein immunocomplexes were analyzed by electrophoresis as in A. The positions of molecular mass markers (MW) and cross-linked HuR in panels A and B are indicated. C, band shift assays using various concentrations of purified recombinant MBP (10^-4 M) or MBP-HuR (10^-6, 10^-7, 3 x 10^-8, 6 x 10^-7, and 10^-6 M) proteins and radioactively labeled RNA sequences corresponding to WT/m0 Fas exon 6. RNA-protein complexes were analyzed on native 6% polyacrylamide gel, dried, and exposed to film. The positions of the free RNAs and RNA-protein complexes are indicated. D, HuR stabilizes U1 cross-linking to the 5′ splice site associated with exon 6. Uniformly labeled WT or m0 RNAs were incubated with control (lanes 1, 3–5, 7, and 8), or U1 knock-out (ko) nuclear extracts (lanes 2 and 6) in the presence of psoralen and in the absence (lanes 1 and 5) or the presence of PTB (50 ng/μl) (lanes 3 and 7) or HuR (50 ng/μl) (lanes 4 and 8) were irradiated with long wave UV light, and RNA was isolated and resolved by electrophoresis in denaturing polyacrylamide gel. The positions of uncross-linked RNA and the product of cross-linking to U1 snRNA are schematically indicated. E, HuR inhibits the U1 snRNP-dependent, 5′ splicing site-dependent cross-linking of U2AF65. Uniformly labeled RNAs corresponding to exon 6 and neighboring sequences (68 to 30 or 68 to the 3′ end of exon 6) were incubated with normal nuclear extracts in the absence (lanes 1 and 6) or presence of PTB (lane 3) or HuR (lane 4) (50 ng/μl), U1-inactivated nuclear extracts (lane 2), or chromatographically depleted extracts of U2AF (ΔU2AF) (lane 5) as indicated. F, HuR does not inhibit U2AF65 cross-linking in the absence of the URE6 (lane 3) but does inhibit it in the absence of 5′ splice site sequences (lane 7). Assays were carried out using RNAs with the URE6 sequence mutated (m0) or lacking intron 6 sequences, as indicated.
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Fas 2xMS2 minigenes, which when present strengthened the intron 6 5’ splice site, intron 5 3’ splice site, or both. In this scenario, the RRM1 domain functioned as a potent repressor of RNA substrates with weak 5’ and 3’ splice sites and with stronger 5’ splice sites but not with stronger 3’ splice sites (Fig. 5C, compare lanes 3 and 7 with lanes 11 and 15). Surprisingly, the hinge-RRM3 fusion was a potent repressor in all conditions examined, even when the 5’ and 3’ splice sites were stronger (Fig. 5C, compare lanes 4 and 8 with lanes 12 and 16). These results point to a dual behavior of the functional domains of HuR splicing repressor and a major role of the hinge-RRM3 domains, which resides the capacity for cooperative assembly of HuR oligomers on RNA, in inhibition of Fas splicing by targeting molecular events that lead to exon definition.

Finally, to demonstrate the functional duality of RRM1 and hinge-RRM3 domains and their decisive contribution to the role exerted by HuR as Fas splicing repressor, I generated these domains as GFP-tagged fusion proteins to test their contribution to the splicing regulation of a WT Fas minigene. These domains were tested as cis- and trans-acting regulatory elements. As shown in Fig. 5D, I did not detect significant differences between the regulatory capacities of domains by themselves, observing dominant-negative effects that might be predicted a priori (Fig. 5D, compare lanes 1 and 2 with lanes 3–6). Overexpression of truncated variants of HuR lacking these domains abolished repressive activity of HuR (Fig. 5D, compare lane 2 with lanes 7 and 8). I conclude that both RRM1 and hinge-RRM3 domains of HuR are necessary to recapitulate the repressor activity of the splicing factor HuR.

**DISCUSSION**

The apoptosis-inducing proteins TIA-1/TLIR promote U1 snRNP recruitment to the 5’ splice site of intron 6, in turn facilitating exon definition by enhancing U2AF binding to the 3’ splice site of intron 5 and, therefore, Fas exon 6 inclusion (10). I show that HuR promotes exon 6 skipping through an exon splicing silencer, URE6, by inhibiting molecular events leading to exon definition via stabilization of U2AF65 binding (Fig. 6). These findings provide a functional link between HuR, a novel Fas splicing regulator, and molecular events leading to exon definition.
HuR Targets Exon Definition—Hu proteins (HuB, HuC, and HuD) have been recently identified as splicing regulators in neuron-like cells (15). Such proteins can directly block TIA-1 activity on neuron-specific alternative RNA processing of the calcitonin/calcitonin gene-related peptide pre-mRNA (15). Here, I report for the first time that HuR, the only ubiquitously expressed Hu protein in mammalian cells, is a novel splicing regulator with a putative wide range of action. My results show that HuR operates differently to other Hu proteins (15). The repressive effect of HuR on Fas exon 6 splicing does not require 3’ of the URE6 element to prevent U1 snRNP and/or TIA-1/TIAR interactions with the 5’ splice site of exon 6 and downstream sequences. HuR-mediated inhibition interferes with the molecular interactions that lead to exon definition (3, 37). I reported previously that recognition of the 3’ splice site of Fas exon 6 is strongly dependent on exon definition effects and that PTB/Raver-1 can inhibit the bridging interactions that mediate the events leading to exon definition (10). The results in Fig. 4 strongly suggest that HuR acts by interfering with the molecular bridges between U1 snRNP and U2AF65 that lead to exon definition and by direct competition of U2AF65 binding to the 3’ splice site. This double inhibitory effect is exerted through the RRM1 and hinge-RRM3 functional domains of HuR (Fig. 5, B–D). Fialcowitz-White et al. (36) recently demonstrated that the RNA binding affinity of HuR is RRM1–2-dependent, whereas the capacity for cooperative assembly of HuR oligomers on RNA resides in hinge and RRM3 domains (36). I therefore propose that multimerization of HuR, favored by hinge and RRM3 domains, interferes with molecular interactions leading to exon definition and U2AF65 recruitment. Nevertheless, it is still unclear how HuR RRM1 functions as a repressive domain of Fas splicing. It is tempting to suggest that this domain per se may also counteract the bridging interactions leading to exon definition by means of another unknown mechanism. However, consistent with the repressive effects of both domains, RRM1 and hinge-RRM3 fragments of HuR function as trans-acting dominant-negative proteins in an independent manner (Fig. 6). By analogy with the mechanisms operating for other splicing repressors, HuR might partially function like hnRNP A1 (40) or PTB (41). HuR binding to a high affinity site located into URE6 sequence could trigger multimerization on nearby sequences that would prevent or displace the association of factors binding to juxtaposed enhancers and/or splice sites (Fig. 6). My combined results indicate that this model is likely to operate in the regulation of Fas exon 6.

Potential Physiological Consequences—My results support an interesting link between regulation of alternative Fas splicing by HuR and apoptosis in cancer cells. In vivo, HuR overexpression is observed in many human cancers, including brain, breast, colon, lung, and ovary tumors (27). I found that overexpression of HuR in HeLa cells, a cervical carcinoma cell line, leads to Fas exon 6 skipping. The finding that, like TIA-1, TIAR, PTB, FASTK (Fas-activated serine/threonine kinase), and SPF45 (splicing factor 45 kDa) (7, 10, 34, 42), HuR is also involved in the alternative splicing control of Fas exon 6 suggests that the switch in expression between pro- and antiapoptotic Fas isoforms is tightly regulated. I suggest that HuR overexpression might be a strategy for tumor cells to escape apoptosis. This suggestion agrees with the role assigned to HuR as an antiapoptotic and survival factor since this protein can be the master regulator of the coordination of a post-transcriptional antiapoptotic program in human cells (23). The network of HuR interactions might have a more widespread function in the regulation of additional alternative splicing events. Given that about 74% of human pre-mRNAs have alternative isoforms (2), more primary transcripts may be found that can be alternatively spliced through the action of HuR.

HuR is emerging as a key regulator, a new paradigm, of the post-transcriptional regulation in mammals. Given that the same RNA-binding protein can regulate several aspects of gene expression under different environmental conditions, it is interesting to note that a multifunctional protein such as HuR may be involved in coupling different molecular events affecting the post-transcriptional control, including splicing, transport, localization, stability, and translation. HuR is predominantly nuclear under normal growth conditions but shuttles between the nucleus and the cytoplasm through a specialized domain, the shuttling sequence HNS (for HuR nucleocytoplastic shuttling) (16). HuR regulates RNA metabolism events in the nucleus and cytoplasm. In the nucleus, HuR functions as a pre-mRNA splicing regulator, probably as a specific repressor of exon inclusion, like PTB (44), thanks to its capacity to bind to adenosine- and/or uridine-rich tracts located in intronic and exonic sequence elements on the pre-mRNA (this study). Thus, HuR may be regulating other splicing events affecting many relevant genes to control key cellular processes. In the cytoplasm, HuR is involved in the regulation of stability and translation of target mRNA subsets (45). In most systems regulated by HuR, control is achieved through interaction of HuR with multiple HuR binding sites surrounding the target sequence located at the 3’-untranslated regions of the cellular mRNAs (36). Furthermore, HuR post-translational modifications (including phosphorylation by Chk2 (22)) or its cellular compartmentalization could prevent or reverse its regulatory features. In summary, HuR seems to use multiple molecular mechanisms to modulate gene expression at the post-transcriptional level, showing that the control of human genome expression involves complex networks of epigenetic interactions.

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Addendum—Interestingly, while this report was under revision, Zhu et al. (43) reported that neuron-specific Hu proteins control the production of the short protein isoform from neurofibromatosis type I (NF1) pre-mRNA by suppressing inclusion of NF1 exon 23a, whereas TIA-1/TIAR proteins promote inclusion of this exon. Mechanistically, they identify two binding sites for Hu proteins, located upstream and downstream of the regulated exon, and provide biochemical evidence that Hu proteins specifically block exon definition by preventing binding of essential splicing factors. In fact, these proteins prevent binding of U1 and U6 snRNPs to the 5’ splice site of

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NF1 exon 23a and decrease U2AF binding to upstream 3′ splice site, interfering the molecular events that lead to exon definition. Additional studies of HuR as well as other Hu proteins and their target pre-mRNAs will provide relevant insight into the regulatory mechanisms of alternative splicing.

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