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Commentary

Why is Hu where? Shuttling of early-response-gene messenger RNA subsets

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Post-transcriptional regulation of gene expression involves multiple checkpoints at which decisions are made concerning the fate of each messenger RNA species. Primary transcripts assemble into multicomponent ribonucleoprotein complexes during pre-mRNA processing in the spliceosome, are transported out of the nucleus, and are either stored, degraded, or translated in the cytoplasm (1). Determining the outcome of this process for each individual mRNA species is complicated by the heterogeneity of the messenger ribonucleoprotein (mRNP) population, which contains various-size transcripts and diverse combinations of attached proteins. Although investigators have not yet devised methods to separate and characterize distinct cellular mRNPs, nature appears to distinguish each mRNP by using inherent signals in the RNAs and/or in the proteins in the mRNP complexes and tracking each mRNA to an appropriate functional outcome. For example, mRNAs may contain recognition sequences that can serve as "zip codes" for tracking or localization (2) whereas in other cases, proteins attached to the mRNPs contain localization signals that allow their movement out of, or into, the nucleus (reviewed in ref. 3). In a previous issue of the *Proceedings*, Fan and Steitz (4) define a novel nuclear shuttling sequence in the Hu RNA-binding protein HuR, which may transport a specific subset of cellular mRNAs containing AU-rich elements (ARE) from the nucleus to the cytoplasm (Fig. 1). They term this signal the "HNS" for HuR nuclear-cytoplasmic shuttling sequence because it contains both nuclear export and nuclear localization elements. This is particularly interesting because the ARE-containing mRNAs encode a functionally important subset of early-response gene (ERG) or immediate-early gene products, including protooncoproteins and cytokines.

HuR (also called HuA) is one of four members of a family of human proteins that are highly homologous to a *Drosophila* nuclear protein known as ELAV (pronounced "e-la-vee"), for embryonic lethal abnormal vision. Deletion mutants of the *elav* gene are embryonic-lethal because of abnormal development of neurons (5, 6). Temperature-sensitive mutations result in abnormal neuronal differentiation, which is most apparent in defects in the formation of Drosophila eyes. In mammals and in *Xenopus*, the Hu family consists of three members that are developmentally regulated and tissue-specific [Hel-N1 (also called HuB), HuC, and HuD], and one (HuR) that is ubiquitously expressed in all cell types (7–11). The mammalian ELAV/Hu cDNA encoding Hel-N1 (10) and all four *Xenopus* ELAV cDNAs (7) were cloned by using degenerate PCR based on Drosophila ELAV sequences whereas HuR was derived by degenerate PCR based on HuD cDNA sequences (8, 11). The mammalian HuC and HuD cDNAs were derived by screening a cDNA expression library with Hu autoimmune serum (11). The autoimmune serum used for the expression-cloning of HuD came from a patient with a paraneoplastic neurological disorder (PND) (12–14).

PND patients have certain types of cancers, predominantly small cell lung carcinoma, and, in other cases, breast, ovarian, or prostate cancer. During the course of their disease, they develop autoantibodies against proteins ectopically expressed in the tumors (12). The humoral and cellular responses are mounted against these tumor proteins because they are normally expressed in an immune privileged site such as the central nervous system. In the case of the ELAV/Hu proteins, the small cell lung tumor expresses a tissue-specific Hu antigen: Hel-N1, HuC, or HuD. The antibodies made in response to the tumor antigen, as well as inflammatory cells, cross the blood–brain barrier, resulting in PND-associated encephalomyelitis and neuronopathy (12, 13). One fascinating observation is that the tumors in PND patients remain small as a result of the tumor-specific immune response, and the patients die of neuronal degeneration rather than cancer (14, 15). This immune suppression of tumor growth in PND patients could be viewed as biological validation of ELAV/Hu proteins as potential therapeutic targets. As discussed below, the functions of ELAV/Hu proteins in up-regulating the expression of ERG mRNAs and their gene products is consistent with this notion.

Backed by the strong genetic evidence that the fly *elav* gene is critical for neuronal differentiation (5, 6), the next clue to the function of the mammalian ELAV/Hu proteins came from the selection of RNAs from combinatorial libraries. These experiments indicated a strong preference for binding to AREs found in the 3′ untranslated regions of ERG mRNAs (16, 17). The ARE-binding specificity of ELAV/Hu proteins was later confirmed for all of the four mammalian family members (reviewed in ref. 9). ARE sequences first were recognized as important in 1986, when Shaw and Kamen (18) and Caput *et al.* (19) noted that protooncogene and cytokine mRNAs have characteristic AU-rich sequences, best defined by a pentamer, AUUUA, in their 3′ untranslated regions. ARE sequences were shown to embody a critical aspect of instability of ERG mRNAs (ref. 18 and reviewed in refs. 20 and 21). Instability sequences are believed to help keep ERG mRNAs in constant flux so the cellular output of growth regulatory proteins can respond rapidly to environmental signals or to changes in transcription. Thus, highly stable mRNAs are buffered against rapid changes in transcription whereas the levels of unstable mRNAs can change rapidly as more or less mRNA is transcribed. During growth regulation, cell cycle, and differentiation, RNA stability is believed to be an important regulatory mechanism (21). A classical example is the immediate-early response of c-fos transcription, in which addition of serum to serum-depleted cells causes exit from the G_0 phase of the cell cycle and entry into G_1. The rapid and transient increase and subsequent decrease in the level of c-fos mRNA are possible because c-fos mRNA is turned over rapidly in the cytoplasm (20, 21). It should be noted that other non-ARE instability elements have been identified in coding regions of some ERG mRNAs, including that of c-fos (21).

The biological relevance of ARE-mediated mRNA stability in proliferation and differentiation is best exemplified in the immune system. For example, after T cell activation, ARE-mediated stability is a fundamental mechanism regulating cytokine mRNA levels in the cytoplasm (22). Of interest, levels of

The companion to this Commentary begins on page 15293 in issue 26 of volume 95.

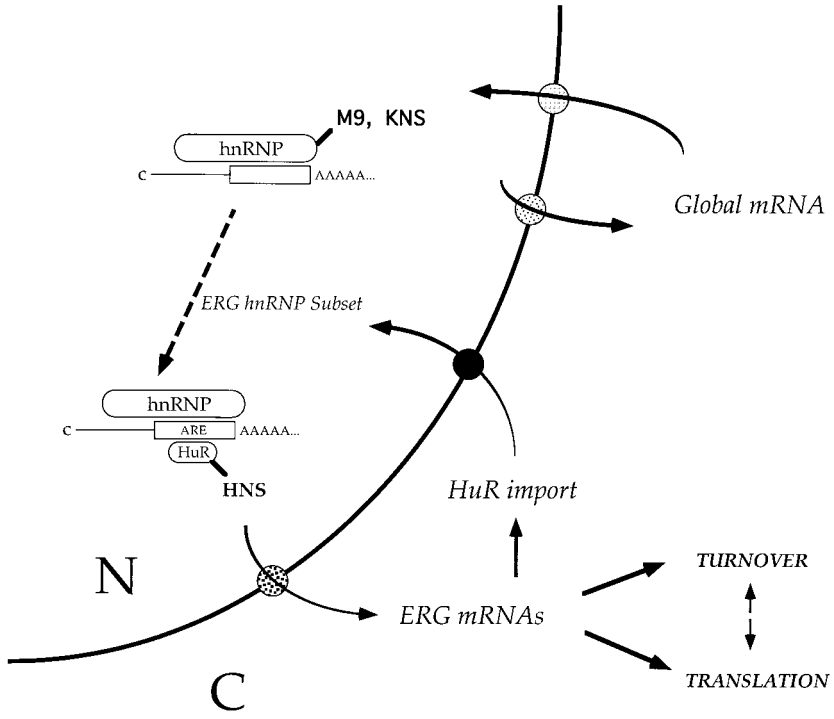

FIG. 1. Nuclear-cytoplasmic shuttling of the ELAV/Hu protein HuR, which binds to a distinct subset of cellular messenger RNAs. ERG mRNAs encoding protooncoproteins and cytokines are bound to and stabilized by HuR, which contains an amino acid segment (HNS) with both nuclear export and import elements (4). On reaching the cytoplasm, HuR is assumed to release the ERG mRNAs for degradation or translation and may then return to the nucleus (HuR-import) or degrade. Nuclear pores through which mRNPs and proteins may pass are represented as filled circles. The tension between these competing localization signals results in a steady-state distribution of HuR between the nucleus and cytoplasm and may determine the amounts of ERG mRNAs available for translation. ERG mRNAs are assumed to be a subset of the global heterogeneous mRNA population. The hnRNP proteins complexed with ERG mRNAs may contain other nuclear-cytoplasmic shuttling signals (41, 42).

mouse HuR increase dramatically in activated T cells and during periods of increased proliferation of cultured 3T3 cells (23). However, it is likely that ERG mRNAs can be regulated independently of one another during biological responses such as T cell activation or that they are regulated as subsets of the larger ARE-containing population. It is assumed that many transacting proteins are involved in the stability and translation of individual ERG mRNAs. Another developmental process in which an immediate early response is associated with mRNA turnover and/or translational control of ERG mRNAs is neuronal plasticity (25, 26). Consistent with this possibility, the ELAV protein, Hel-N1, is expressed most highly in the hippocampus of rat brain (10), an area of active neuronal plasticity; also, Hel-N1 was found to increase dramatically during neuronal differentiation (9, 27). Although these correlations are intriguing, the extent to which ELAV/Hu proteins participate in cell proliferation, immune cell activation, and neuronal plasticity is yet to be determined.

The ARE represents one of the few identified sequences in cellular mRNAs that defines a distinct structural and functional subset (18–21). Therefore, an intense effort has ensued to identify proteins that bind ARE-containing mRNAs or function as transacting factors to modulate their stability or regulate their expression. Of the several ARE-binding proteins identified, only the ELAV proteins have been found to date to affect the stability and/or translatability of ERG mRNAs, as mediated by the ARE (9, 28–31). Both the Shenk (32) and the Steitz (33) groups observed that a predominantly nuclear, 32-kDa protein could be UV-crosslinked to ARE-containing mRNAs, and they both predicted that the protein would participate in ARE-mediated rapid degradation. Myer *et al.* (34) demonstrated that the 32-kDa protein was HuR (8), the precise homolog of the ubiquitously expressed *Xenopus* ELAV protein, elr-A (7). Given the strong binding preference of ELAV proteins for ARE sequences, Jain *et al.* (28) reported that ectopic expression of Hel-N1 in 3T3-L1 cells led to stabilization of an endogenous ARE-containing mRNA encoding the glucose transporter 1 (GLUT1) protein. In addition, the levels of GLUT1 protein increased dramatically in transfected cells after induction of 3T3-L1 cell differentiation. This was the first indication that an ARE-binding protein could lead to increased stability, rather than instability of a target mRNA. Stabilization of c-fos and GM-CSF ARE-reporter transcripts, as well as the vascular endothelial growth factor mRNA, subsequently was shown by transfection of HuR cDNA into various cultured cells (29–31). The ability of ELAV/Hu proteins to stabilize ARE-containing mRNAs was confirmed recently with both Hel-N1 and HuR proteins by using an *in vitro* deadenylation/degradation system (35). In addition to its effect on GLUT1 mRNA stability, Jain *et al.* (28) also reported that Hel-N1 expression caused GLUT1 mRNA to engage with active polysomes, suggesting a translational effect in addition to its effect on mRNA stability. Many reports have suggested an intimate link between mRNA translation and mRNA stability involving ARE-containing unstable mRNAs (reviewed in ref. 36; ref. 37), although the two processes can be uncoupled (38).

Although genetic evidence is not available in the mammalian system, data from transfection studies and from differentiation of cultured cells strongly suggest that differentiation-specific genes can be activated by or in concert with the appearance of ELAV proteins (9). As mentioned above, ectopic expression of Hel-N1 in 3T3-L1 cells results in the uptake and storage of lipid in conjunction with stabilization and increased translation of GLUT1 mRNA (28). This gave rise to the appearance of an apparent enhanced differentiation phenotype after treatment with insulin. Moreover, after induction of neuronal differentiation of P19 embryonic carcinoma cells by using retinoic acid, Hel-N1 is up-regulated dramatically whereas Hel-N2, a form that is associated with growing cells, is down-regulated (17, 27). Hel-N2 lacks a 13-aa segment in a "hinge" region between two of three RNA recognition motifs of Hel-N1 (refs. 17 and 27; see Fig. 6 of ref. 4 in this volume). This transition involves an alternative splice and results in a complete conversion of Hel-2 to the terminally-differentiated neuronal form Hel-N1 (ref. 27; N. Lu, D. Antic, and J.D.K., unpublished work). Of interest, Hel-N1 and Hel-N2 are expressed in a variety of tumors and cell types, including testes and ovaries (10, 16, 17, 27). Transfection of Hel-N1 or HuD cDNAs into neuronal precursor cells results in the appearance of differentiated phenotypes and expression of neurofilament proteins that are characteristic markers of neuronal differentiation (refs. 9, 39, and 40; D. Antic, N. Lu, and J.D.K., unpublished work). This is coincident with the appearance of neurite-like processes in these cells. In the case of Hel-N1, it is believed that these morphological changes result from up-regulation of neuron-specific proteins such as neurofilament M protein, which is induced by interactions between the ectopic

ELAV protein and the NF-M mRNA. Because NF-M is a relatively stable mRNA and contains an atypical ARE to which Hel-N1 binds, the up-regulation of NF-M protein and formation of neurite-like processes is thought to occur at the level of translation rather than stability.

Studies of the intracellular localization of mammalian ELAV/Hu proteins presents several enigmas that are addressed, in part, by the report from Fan and Steitz (4). For example, it was a mystery as to how a nuclear protein like HuR could regulate mRNAs that presumably affect stability and translation in the cytoplasm (20, 21) until it was shown to redistribute between these cell compartments (23, 30, 31). Also, the tissue-specific proteins Hel-N1, HuC, and HuD appear to be mainly cytoplasmic in cultured cells, with only a small amount of protein detectable in the nucleus (27, 39). The distribution of ELAV proteins varies among cell types, with tumors containing mostly cytoplasmic ELAV, whereas in neurons from the hippocampus and neocortex, the detectable ELAV proteins appear to be distributed equally in both compartments (reviewed in ref. 9). Of interest, it has been found that the nuclear ELAV proteins appear not to be associated with large complexes whereas the cytoplasmic ELAV exist in two forms: mRNPs bound to polysomes and mRNPs associated with the cytoskeleton (39). It was proposed that ELAV proteins may bind the ARE-containing mRNAs in the nucleus and transport them to the cytoplasm, where they either find access to the translational apparatus or are released for rapid degradation (27, 39). This model has not been tested directly but was surmised from studies of intracellular localization and by using inhibitors of translation and cytoskeletal formation (39). The studies showing that HuR is not exclusively nuclear but can move between the nucleus and the cytoplasm were the first experiments demonstrating a potential role for an ELAV/Hu protein in mRNA transport (23, 30, 31). Although all of these studies used inhibitors of transcription to demonstrate transcription-dependent relocalization of HuR to the nucleus, Fan and Steitz (30) also demonstrated by using heterokaryons of human and mouse cells that HuR could move from the nucleus of one cell through the cytoplasm and into the nucleus of the other species.

Among the ELAV/Hu family, the three RNA recognition motifs are each highly conserved whereas the amino termini and the hinge region connecting RNA recognition motifs 2 and 3 are the most diverse (7–11). The variation of the hinge region is most apparent by the appearance of short, 13-aa segments that can disrupt the HNS equivalent region at position 218 of HuR (Fig. 6*B* in ref. 4). Therefore, acquisition of any one of the hinge segments could significantly alter the localization and function of the protein. Although the impact of introducing these alternatively-spliced segments is yet to be assessed, it is possible that ELAV proteins use their respective hinge segments for interaction with other cellular factors. As described above, the transition from Hel-N2 to Hel-N1 that occurs during neuronal differentiation of P19 cells results in the acquisition of a single hinge segment (27). As noted by Fan and Steitz (4), there are sequence differences in the equivalent HNS region of Hel-N1, HuC, and HuD that may affect their localization differently from HuR. If HuR binds to newly transcribed or spliced ARE-containing hnRNPs and assists their export out of the nucleus (HuR-HNS), this would define a discrete pathway of mRNP dynamics involving a specific subset of total global mRNAs (Fig. 1). It would seem important for cells to capture these mRNAs while still in the nucleus, to transport them to key locations in the cytoplasm under carefully regulated conditions, and to pass them off for translation or release them for rapid degradation when the protein products are not needed. The released HuR (HuR-import) may return to the nucleus or may be degraded. Therefore, an ongoing process of shuttling HuR (and possibly other ELAV/Hu proteins) out of and back into the nucleus is proposed to involve this unique HNS sequence (4).

Although the relationship of the HNS to other localization and transport sequences (reviewed in ref. 3) is yet to be defined precisely, it has features resembling the M9 shuttling sequence discovered by Dreyfuss and coworkers in hnRNP-A1 (41). One critical difference is that a conserved residue at position 221 of HuR is a serine rather than a glycine, as found in the M9 sequence of hnRNP-A1 (4, 41). The opposing localization signals in both M9- and HNS-containing proteins direct both the nuclear and cytoplasmic compartments, creating a tension that results in a balance of distribution that may be regulated by interactions with other proteins. For example, it will be interesting to determine whether RNA binding alters the properties of either of these competing localization signals. However, one should note that it has yet to be demonstrated that HuR is actually bound to ARE-containing mRNAs in the nucleus and during transport.

Although it is assumed that hnRNP shuttling signals like the M9 or the KNS (41, 42) assist in the export of most housekeeping mRNAs from the nucleus to the cytoplasm as depicted in Fig. 1, it remains possible that distinct sequence elements like the ARE will be discovered that define other mRNA classes. Thus, diverse combinations of mRNA-binding proteins may shuttle different mRNA subsets and regulate their fates in the cytoplasm. Whether shuttling sequences specific for other mRNA subsets are found, it appears that HuR uses the HNS to shuttle the ERG class of mRNAs to the cytoplasm in response to regulatory signals, where they become stabilized, translated, or rapidly degraded.

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