HuR, an RNA-binding Protein, Involved in the Control of Cellular Differentiation

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Abstract. HuR is an RNA-binding protein that resides predominantly in the nucleus but shuttles to the cytosol carrying mRNAs containing a uridylate-rich region in the 3'untranslated region. Data suggest that HuR is involved in the control of the differentiation process through a tightly regulated selection of mRNAs to be shuttled to the cytosol for translation. In this review, the function of HuR in the differentiation process of preadipocytes is examined in the context of specific mRNAs selected by HuR for translocation to the cytosol.

Currently the United States is experiencing an epidemic rise in the occurrence of obesity in both adults and children. Up to one-third of the American population is obese with at least 300,000 deaths a year attributable to obesity, and the estimated costs of treating obesity being reported as $117 billion in 2001. The incidence of Type II diabetes in the US is increasing at an alarming rate; in 2002, total direct health care costs reached $92 billion. Data compiled by the American Diabetes Association indicate that diabetes is the sixth leading cause of death by disease in the US with approximately 16 million people being afflicted in the US alone. In terms of treatment, there continues to be a substantial need for new diagnostic biomarkers and treatment options (1).

Differentiation is a coordinated process of irreversible cell cycle exit and tissue-specific gene expression. To begin to understand the control of this complex process of differentiation, we have used the 3T3-L1 preadipocyte cell system, in which the cells differentiate in a regulated manner into cells with the morphological and biochemical properties of adipocytes. Adipose tissue plays a critical role with respect to both diabetes and obesity and has become recognized as a dynamic endocrine-like tissue, synthesizing and secreting proteins responsible for regulation of the balance between energy storage and energy expenditure (2-5). If new diagnostic biomarkers are to be identified and new treatment options discovered, the regulation of the differentiation process as well as the maintenance of the differentiated phenotype in this complex tissue must be understood.

Our focus is on the definition of key components and the mechanism of a post-transcriptional regulatory network associated with the differentiation of preadipocytes leading to adipocytes. Through characterization of the interaction of RNA-binding proteins with specific mRNAs, we believe a critical early regulatory process will be defined that encompasses the selection of the mRNA in the nucleus and transport of the message to the cytosol where the binding protein controls both the stability and translational efficiency of the mRNA. Understanding these mechanisms will provide details on an RNA-binding protein involved in a very focused regulation impacting gene expression, RNA trafficking and the determination of phenotype.

Post-transcriptional Regulation of Gene Expression and the Hu Proteins

Messenger RNA export from the nucleus, mRNA turnover, as well as translation initiation are important control points in the post-transcriptional regulation of gene expression. At least in part, control of these processes is exerted through recognition of cis elements in the mRNA by specific binding proteins. Our work has focused on regulation mediated by HuR, an RNA-binding protein identified by its ability to bind to an adenylate-uridylate (AU)-rich element (ARE) of...
sequence AU3A within AU-rich backgrounds (6-10). However, there is much flexibility in the design of the binding site with the major consideration being a uridylicate-rich sequence (11). HuR is a 32-kDa protein that belongs to the Hu/ELAV family (embryonic lethal, abnormal vision) of RNA-binding proteins (12, 13). Unlike the other three family members, HuB, HuC and HuD, which are exclusively neuronal, HuR is more broadly expressed and was originally identified as binding to AREs in the 3’ untranslated regions (3’UTRs) of early response genes such as cytokines and proto-oncogenes (7, 9, 14). Unlike the other Hu proteins, HuR is predominantly found in the nucleus (15), and has been suggested to be involved in the shuttling of early response gene mRNA subsets to the cytosol for translation (7, 16), as well as controlling the stability of a number of mRNAs (8-10, 17-21).

Studies from the Steitz laboratory (22, 23), characterizing the translocation process in HeLa cells, have demonstrated that HuR contains a nucleocytoplasmic shuttling sequence and functions as an adapter protein in the nuclear export of mRNAs that contain an ARE in their 3’UTRs (7, 9, 14). As shown in Figure 1, depending on the specific mRNA and cellular conditions, the nuclear HuR containing messenger ribonucleoprotein (mRNP) may be bound by APRIL and/or pp32 and then by CRM1, which is recognized by a specific binding domain in the nuclear pore complex, facilitating nucleocytoplasmic transport. An alternative to the CRM1 route is mediated by transportin-2 binding to the HuR mRNA complex for exit through the nuclear pore (22, 23). Once in the cytosol, HuR functions to stabilize its mRNA ligand (24, 25). In addition to stabilization, our earlier studies with ectopic expression of HuB would suggest that HuR may also play a role in the control of translation (26-28). The selection of ligands and the translocation of HuR to the cytosol are proposed to be under tight control (29). Identification of the specific signal involved has remained elusive, with the exception of lipopolysaccharide-stimulated macrophages in which methylation of specific arginine residues in HuR by the nuclear CARM methylase appears to control the translocation process (29). In other studies using senescent fibroblasts or human colorectal carcinoma cells, increased AMP-activated protein kinase (AMPK) activity resulted in nuclear retention of HuR (30, 31). Importantly, the authors neither demonstrated nor claimed that HuR is a direct target of AMPK phosphorylation (30, 31). In these cell systems under specific conditions, AMPK activity resulted in nuclear retention of HuR (30, 31).

It is important to realize that the current model for message translocation would suggest that the mRNA ligand of HuR does not leave the nucleus unless it is bound to HuR, and movement of HuR to the cytosol is emerging as a key regulatory function for HuR.

**Hu Proteins, the Cell Cycle and Differentiation**

Among the mRNAs identified as ligands for HuR are several messages encoding cell cycle-regulatory proteins like cyclins A and B (32) and the CDK inhibitor p21Cip1 (33, 34). Binding of HuR to these elements is believed to stabilize the mRNA, therefore leading to increased protein levels, thus facilitating or inhibiting cycle progression dependent on the particular conditions. In addition to the stability function of Hu/ELAV proteins, HuD and HuR have been reported to bind to the 5’UTR of the CDK inhibitor p27Kip1 mRNA and repress expression by reducing its internal ribosomal entry site-dependent translation (35, 36).

The ELAV gene product was discovered to be essential for the development of the *Drosophila melanogaster* nervous system (12, 13). The first demonstration that a Hu/ELAV protein was involved in the differentiation process of mammalian cells came from the work of Jain *et al.* (26). In these studies, cells were transfected with neuronal HuB (Hel-N1) which led to the ectopic overexpression of this neuronal protein in the 3T3-L1 preadipocyte cell line. Relative to parental 3T3-L1 cells, the transfectants expressed a phenotype that led to: i) an acceleration of the differentiation process; ii) an increase in the stability of the mRNA for GLUT1; iii) a marked increase in the rate of translation initiation of the GLUT1 message, but only as the cells differentiated into adipocytes (26). The dramatic effects on the differentiation process evoked through ectopic expression of HuB suggested to us that an endogenous Hu protein may be involved in the attainment of the adipocyte phenotype. That endogenous Hu protein expressed in the 3T3-L1 preadipocytes was identified as HuR.

A role for HuR in the differentiation process was supported by siRNA knockdowns of HuR in C2C12 cells, which resulted in an inhibition of myogenesis coincident with decreased expression of MyoD, myogenin and p21Cip1 mRNAs, known ligands for HuR (37). Recent work (38) provided further support for a link between HuR and differentiation. When the HuR protein content was depleted by siRNA treatment, C/EBPβ, a HuR ligand and a critical early ‘on-switch’ for the differentiation process, was not expressed, resulting in a suppression of the acquisition of the adipocyte phenotype.

With respect to Hu proteins and differentiation, it should be noted that recent studies have supported the roles of HuB, HuC and HuD (but not HuR) in mammalian neuronal cell identity and differentiation (46-48). These data support a role for HuR in muscle and adipose differentiation processes. With the broad expression pattern of HuR, one might expect it to have similar effects on the differentiation of other embryonic cell lines. Such a role for HuR may be unexpected, since differentiation is usually associated with tissue-specific proteins. However, there are
many examples of RNA-binding proteins serving different functions in different tissues (39-45). This could be due to regulated expression levels, protein-protein interactions and/or post-translational modifications, any of which could dictate which mRNA ligand may be selected by HuR. Thus, it can be argued that HuR, by selecting specific mRNA ligands and controlling their expression, is capable of controlling critical events in the cell cycle as well as the differentiation process.

Hu Proteins and Translation

As mentioned above, ectopic overexpression of neuronal HuB in 3T3-L1 cells had a dramatic effect on the translation efficiency and stability of the GLUT1 mRNA (26). This translation/stability enhancement function of HuB was further validated in human teratocarcinoma cells, where overexpression led to a similar increase in the rate of translation initiation of neurofilament M mRNA (27). These two studies were the first to demonstrate the role of the Hu proteins in the regulation of translation. In a follow-up report to that study, Gantt et al. (28) demonstrated that ectopically-expressed neuronal HuB remained predominantly nuclear in the preadipocytes (90% nuclear: 10% cytosolic), establishing a new distribution ratio as the cells differentiated into adipocytes (60% nuclear: 40% cytosolic). Moreover, in the adipocytes, electron micrographs demonstrated that cytosolic HuB localized to polysomes, which is consistent with a role in the translation process as well as potentially mediating the stability of the mRNA. No co-localization of HuB with polysomes was observed in preadipocytes, consistent with a differentiation-specific role for this Hu protein.

The expression of C/EBPβ was also determined (a transcription factor expressed early and transiently in the differentiation program). Interestingly, C/EBPβ mRNA is translated from multiple in-frame AUG sites with the...
potential to give rise to proteins of 35, 32 and 20 kDa species. The p32 and p20 are the predominant forms expressed. p20 C/EBPβ (LIP, liver-inhibitory protein) lacks the transactivation domain and functions as a dominant negative inhibitor of p32 C/EBPβ (LAP, liver-activating protein) as well as the other C/EBPs (49, 50). The LAP to LIP ratio changes during the time-course for differentiation, consistent with the selection of alternative initiation sites being a regulated process and has been suggested to play a role in the control of adipogenesis (49, 50). In studies with the HuB transfectants, Gantt et al. observed a marked increase in the LAP to LIP ratio, suggesting that ectopically-expressed neuronal HuB was influencing the selection of the initiation codon in the C/EBPβ mRNA relative to the parental 3T3-L1 cells (28). Calkhoven et al. (51), working in COS-1 cells transfected with C/EBP constructs, suggested that the LAP to LIP ratio was controlled by a conserved upstream open reading frame (uORF) in the C/EBPβ mRNA and the availability and activity of the eukaryotic translation initiation factors eIF2α and eIF4E. Overexpression of these rate-limiting initiation factors enhanced expression of LIP (the truncated form) of C/EBPβ. Pathways that activate translation favor production of LIP and lead to proliferation and commitment, while pathways that decrease eIF activity lead to proliferation arrest and terminal differentiation. While it is not clear how modulation of eIF activity or availability results in selection of translation initiation sites, it probably involves interaction with tissue- or cell-specific mRNA-binding proteins. In adipose cells, we propose that HuR is involved in the selection of alternative start sites for translation initiation. This resulted in an inhibition of events associated with the differentiation cocktail-induced entry into S-phase of the cell cycle, including: 1. turnover of p27Kip1, an important cdk2 inhibitor; 2. expression of cyclin A and cdk2; 3. DNA replication; 4. MCE and adipogenesis (57). In the context of the importance of C/EBPβ to the differentiation process, Gantt et al. (38) demonstrated: i) constitutive expression of the RNA-binding protein HuR in the 3T3-L1 preadipocytes with a 3-fold increase in protein content as the cells differentiate; ii) at confluence, the majority of HuR protein is retained in the nucleus in the preadipocyte; iii) on exposure of the cells to the differentiation inducers, there is a rapid formation of a nuclear HuR-C/EBPβ complex followed by a translocation of the complex to the cytosol; iv) when HuR expression was reduced (>80%) using siRNA the cells retained their preadipocyte morphology, failed to express normal levels of C/EBPβ and did not accumulate lipid droplets through a 5-day time-course. The authors concluded that HuR protein expression was necessary for the expression of C/EBPβ and progression of the cells through the differentiation process. Work by others has demonstrated that the differentiation process can be blocked with the reagents leptomycin B (72) or AICAR (73). However, in both studies, while differentiation was blocked, the C/EBPβ protein was expressed at normal (72) to above normal levels (73). This suggests that suppression of HuR expression results in an inhibition of differentiation at a step earlier than the accumulation of C/EBPβ protein. The data of Gantt et al. (38) are consistent with that step involving the HuR-mediated movement of the C/EBPβ message to the cytosol. The major pathway proposed to mediate the cytosolic translocation from the nucleus involves recognition of the exon-junction complex by adapter proteins which, in turn, are recognized by specific nucleoporins (6, 22, 74). However, the C/EBPβ gene has no introns and must utilize

### Differentiation, C/EBPβ and HuR

When confluent 3T3-L1 preadipocytes are treated with differentiation inducers, they synchronously re-enter the cell cycle and undergo approximately 2 rounds of cell division as they enter a process that has become known as mitotic clonal expansion (MCE) (53-59). This increase in cell number and commitment to differentiation mimics the increase in adipose mass in obesity that is derived from an increase in both the size and number of adipocytes (60-62). In the 3T3-L1 preadipocytes, mitotic clonal expansion has been demonstrated to be required for terminal adipocyte differentiation (53-59) and a transcription factor expressed prior to the start of the process, C/EBPβ, plays an essential role in MCE as well as subsequent events in the differentiation program (53-59, 63). Transcription of the C/EBPβ gene is increased shortly after exposure of the cells to the differentiation inducers and, within 4 hours, C/EBPβ protein can be detected in the nucleus (64). However, acquisition of C/EBPβ DNA-binding activity is delayed (16 h), concurrent with entry into the S-phase at the onset of MCE and the transactivation of the C/EBPβ and PPARγ genes by C/EBPβ (56). This lag has been suggested to be necessary as both C/EBPβ and PPARγ are antimitotic and premature expression would inhibit the MCE and, thus, block differentiation. As C/EBPβ expression is increased, C/EBPβ expression is attenuated and the adipocyte phenotype is expressed (65-71). Support for the primacy of C/EBPβ in both MCE and the differentiation process was demonstrated by expression of a dominant negative C/EBPβ which interfered with C/EBPβ function after induction of differentiation. This resulted in an inhibition of events associated with the differentiation cocktail-induced entry into S-phase of the cell cycle, including: 1. turnover of p27Kip1, an important cdk2 inhibitor; 2. expression of cyclin A and cdk2; 3. DNA replication; 4. MCE and adipogenesis (57). In the context of the importance of C/EBPβ to the differentiation process, Gantt et al. (38) demonstrated: i) constitutive expression of the RNA-binding protein HuR in the 3T3-L1 preadipocytes with a 3-fold increase in protein content as the cells differentiate; ii) at confluence, the majority of HuR protein is retained in the nucleus in the preadipocyte; iii) on exposure of the cells to the differentiation inducers, there is a rapid formation of a nuclear HuR-C/EBPβ complex followed by a translocation of the complex to the cytosol; iv) when HuR expression was reduced (>80%) using siRNA the cells retained their preadipocyte morphology, failed to express normal levels of C/EBPβ and did not accumulate lipid droplets through a 5-day time-course. The authors concluded that HuR protein expression was necessary for the expression of C/EBPβ and progression of the cells through the differentiation process. Work by others has demonstrated that the differentiation process can be blocked with the reagents leptomycin B (72) or AICAR (73). However, in both studies, while differentiation was blocked, the C/EBPβ protein was expressed at normal (72) to above normal levels (73). This suggests that suppression of HuR expression results in an inhibition of differentiation at a step earlier than the accumulation of C/EBPβ protein. The data of Gantt et al. (38) are consistent with that step involving the HuR-mediated movement of the C/EBPβ message to the cytosol. The major pathway proposed to mediate the cytosolic translocation from the nucleus involves recognition of the exon-junction complex by adapter proteins which, in turn, are recognized by specific nucleoporins (6, 22, 74). However, the C/EBPβ gene has no introns and must utilize
an alternative export mechanism, which is probably mediated by HuR recognition of the ARE in the 3′UTR (22). This selection of the C/EBPβ mRNA by HuR and mediation of its translocation to the cytosol becomes a potentially critical control step in the onset of adipogenesis. In addition, these data are consistent with other models, where cytosolic translocation of Hu proteins and their associated mRNAs correlated with the onset of myogenensis or neural development (37, 74). Previous studies demonstrated that ectopic overexpression of neuronal HuB in the 3T3-L1 preadipocytes resulted in an early expression of C/EBPβ followed by a rapid onset of differentiation and increased deposits of triacylglycerol (26, 28). These data provide support for the premise that an endogenous member of the Hu family is involved in the temporal control of the differentiation process. The data of Gantt et al. (38) are consistent with HuR serving this purpose.

The work of Gantt et al. (38) has demonstrated that the choice of a ligand mRNA by HuR is a selective process (38). Neither Cyclin D1, an ARE-containing mRNA expressed with the same kinetics as C/EBPβ (74), nor GATA3, a message without an ARE, were present in the mRNP complexes (38). Interestingly, recombinant HuR will bind to the Cyclin D1 ARE in an RNA gel shift assay. However, HuR present in extracts prepared from serum-stimulated RKO cells did not bind to the Cyclin D1 ARE in similar gel shift assays (32). These data are consistent with either the metabolic/hormonal state of the cell controlling the selection of ligands by the HuR protein or other RNA-binding proteins competing effectively for the HuR-binding site. We would suggest that the tight temporal control of specific mRNA selection and translocation to the cytosol during the differentiation process would be an absolute necessity and that these data support the existence of a new control point in the differentiation process.

As the cells express the adipocyte phenotype, a larger proportion of the HuR protein is found in the cytosol per unit time long after expression of C/EBPβ expression has diminished, with the expectation that HuR is still actively shuttling between nuclear and cytosolic compartments and having demonstrated that other ligand mRNAs are selected with respect to the time-course of differentiation (75, and Gantt, Atasoy and Pekala unpublished data). We suggest that HuR not only functions in establishing the adipocyte phenotype, but aids in its maintenance.

Our hypothesis is that HuR participates in the post-transcriptional control of the expression of gene products necessary to initiate and maintain the adipocyte phenotype. While C/EBPβ may be a primary target, other critical mRNAs, i.e., cell cycle-related, may also play a role. In addition, once the master-switch is flipped, HuR participates in regulating the expression of genes necessary for maintenance of the adipocyte phenotype. We believe that this control point in the initial stages of the differentiation process may serve as a potential drug target to block the differentiation process and, thus, a potential treatment option for obesity.

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