

Up to the minute project summary:

As preadipocytes differentiate, temporally controlled expression of C/EBP β is essential to acquisition of the adipose phenotype. Transcriptional activation of C/EBP β in the 3T3-L1 cells occurs within minutes of exposure to the differentiation inducers and is controlled by the cAMP response element binding protein (CREB). In the normal differentiation program of 3T3-L1 cells, C/EBP β first controls the entry of the cells into mitotic clonal expansion, then the expression of C/EBP α and PPAR γ . The timing of expression during these processes is critical as C/EBP β is pro-mitotic and C/EBP α is anti-mitotic and thus C/EBP β expression must attenuate as C/EBP α expression initiates. Highly controlled expression of C/EBP β is essential for proper acquisition of the adipocyte phenotype. The studies conducted through ADA funding describe an early level of posttranscriptional regulation initiated in the nucleus that appears to control the regulated entry of the C/EBP β mRNA into the cytosol and/or the availability of C/EBP β mRNA for translation.

The C/EBP β mRNA contains an adenylate – uridylate rich region (ARE) between bases 1291–1400 of the 3'UTR, a motif that, when present, confers instability to the message. Our previous work has demonstrated that this ARE and hence the C/EBP β message is a ligand for the RNA binding protein HuR, a protein that when bound stabilizes its ligand message. In the 3T3-L1 cells HuR is constitutively expressed and localized predominantly to the nucleus in the preadipocytes. However within 30 min of exposure to the differentiation stimulus, the HuR content in the cytosol increases, consistent with HuR regulating the availability of relevant mRNAs for translation. Within 30 min of initiation of the differentiation process, HuR complexes containing C/EBP β mRNA selectively formed in the nucleus, the complex then appeared to translocate to the cytosol. The proximity of this event to the induction of differentiation and the importance of C/EBP β to the differentiation process led us to hypothesize a role for HuR in the regulation of the onset of adipogenesis. Further support for this hypothesis was obtained in our study when siRNA suppression of HuR protein content resulted in an inhibition of C/EBP β protein expression as well as the downstream targets of C/EBP β transactivation, C/EBP α and PPAR γ . These regulations led to an attenuation of the differentiation process. The hypothesis that we developed proposes that HuR expression appears to be required for differentiation. However while our data demonstrate a specific interaction between HuR and the C/EBP β message, it does not suggest that the C/EBP β message is the only ligand for HuR or mandate that the suppressive effect on differentiation observed when HuR expression was diminished is solely due to the effect on C/EBP β .

In the above study, the identification of a HuR binding site in the C/EBP β mRNA was accomplished by inspection and use of the COVELS algorithm which predicted a single HuR binding site between bases 1370 and 1386 of the 3'UTR. This region was confirmed to bind HuR through *in vitro* RNA gel shift analysis as well as immunoprecipitation of HuR containing messenger ribonucleoprotein particles and RTPCR analysis of the bound message. This region exhibits a high A+U content and would be expected to serve as an ARE, conferring instability to the message. However, a recent report demonstrated that in dendritic cells HuR could function to mediate translocation from the nucleus to the cytosol of the mRNA coding for the cell surface protein CD38. Interestingly, the CD38 mRNA does not contain an ARE and HuR was demonstrated to bind to a structured RNA element localized to the coding region of this message. Based on this description of a novel HuR binding site coupled to a translocation function we initiated an examination of the entire C/EBP β mRNA for non ARE related HuR binding sites. The data obtained support the existence of a single site in the C/EBP β mRNA for HuR binding which corresponded to the ARE in the 3'UTR, which we had previously defined.

We then asked the question as to the importance/function of the HuR-C/EBP β mRNA interaction. The approach was based on the work of Steve Farmer and colleagues in which conditional ectopic expression of C/EBP β in the multi-potential NIH 3T3 cells in the presence of the differentiation induction cocktail, induced PPAR γ and stimulated adipogenesis. In our current study, we are comparing the phenotype expressed when NIH-3T3 cells were transfected with constructs expressing a wild type C/EBP β and a variant in which the HuR binding site was

deleted and replaced with a series of bases that did not bind HuR (β d/s; for ~~delete~~/substitute). Unexpectedly, the β d/s expressing cells accumulated more lipid and appeared to have an enhanced differentiation program. The observation suggested that removal of the capacity for C/EBP β to bind HuR resulted in the loss of a control or restraint on the differentiation process. At the level of gene expression, C/EBP β protein was markedly more abundant in the β d/s cells than in those expressing the wild type β . Expression of C/EBP α was significantly elevated above the wild type cells consistent with the elevated level of C/EBP β driving C/EBP α expression. Interestingly, PPAR γ expression was identical in both wild type and β d/s cell lines suggesting that even the lower level of C/EBP β expressed in the wild type cells was sufficient to drive maximal expression of PPAR γ . Thus, the strong expression of both C/EBP β and C/EBP α combined with expression of PPAR γ was responsible for the robust onset of the adipocyte phenotype in the β d/s cell line.

The question becomes why did loss of ability to bind HuR lead to overexpression of C/EBP β ? Examination of both wild type and d/s cell lines indicated that the ***total C/EBP β mRNA*** was present at identical levels. However when the cytosolic component was examined, the amount of C/EBP β mRNA present in the d/s cell line was approximately 3 times that of the wild type. Examination of mRNA stability, indicated that the half-lives of the two RNAs were identical suggesting that the increased cytosolic accumulation of the C/EBP β mRNA in the d/s cell line was not due to increased stability on loss of the HuR binding site. The data would suggest that more of the C/EBP β mRNA accumulated in the cytosol of the d/s cells because it appeared to translocate from the nucleus to the cytosol more readily than the HuR binding wild type message. The results with the β d/s construct demonstrate that binding of HuR to the ARE is not necessary for translocation from the nucleus to the cytosol and are consistent with HuR binding functioning as a restraint, providing a regulated entry of the message into the cytosol. The tightly controlled entry of the C/EBP β message into the cytosol and the translation apparatus would lead to a well regulated onset of adipogenesis. Based on these observations we suggest that at least in some cases, alterations in the ability of HuR to bind the C/EBP β mRNA could lead to an unregulated differentiation and result in the onset of obesity.

In addition to describing a new regulatory step in the differentiation process, these data further examine the role for HuR in nuclear transport. HuR has a predominantly nuclear localization and can shuttle between nucleus and cytoplasm. This shuttling may have a functional role as HuR has been shown to serve as an adaptor for the nuclear export of a class 1 ARE mRNA, c-fos. C/EBP β has a class 3 ARE; it is an unstructured uridylyte rich region and it is not clear whether HuR mediated shuttling is of functional significance with this class of ARE. While we have previously demonstrated that a nuclear complex between HuR and the ARE present in the C/EBP β mRNA forms within 30 min of induction of differentiation, the function or necessity of complex formation for translocation remains unknown. Our current data would suggest that somewhere in the pathway between complex formation in the nucleus and active translation of the C/EBP β mRNA in the cytosol there exists a slow step that is controlled by HuR binding. Removal of that control results in more C/EBP β mRNA available for translation, more C/EBP β protein and a more aggressive differentiation.

HuR may not act alone. Previous studies from other laboratories have demonstrated HuR to function as a negative posttranscriptional modulator by synergizing with the translational modulator TIA-1 to reduce the translation of cytokine mRNAs. Yet in another study HuR functioned to promote cytochrome c translation while TIA-1 functioned as a translational repressor. Additionally, there are the microRNAs (miRs), a new class of abundant small RNAs that play important regulatory roles at the posttranslational level; by binding to their target mRNAs they either block their translation or initiate their degradation, according to the degree of complementarity with the target. Recent computational analysis documents the presence of a binding site for miR 155 at nucleotides 1430 -1451 of the C/EBP β 3'UTR, which is proximal to the 3' terminus of the HuR binding site. Perhaps it is an interaction between HuR and TIA-1 and/or miR155 that governs the correct amount of C/EBP β (wild type) mRNA required for a controlled differentiation process and that when the binding interactions are disrupted (β d/s), the C/EBP β mRNA enters the cytosol unregulated leading to overexpression of the protein and a more robust differentiation. However on examination we could not demonstrate the presence of

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a TIA-1 – C/EBP β mRNA interaction. Moreover, miR 155 does not appear to be expressed in either the 3T3-L1 or NIH-3T3 cells.

Recently it was reported that in an *in vitro* assay, HuR binding suppressed the rate and extent of polyadenylation. Suppression of polyadenylation could lead to nuclear retention and decreased translocation of a message to the cytosol. If this was occurring, it would explain the observations described above. With this consideration we have proposed that when HuR is bound to the C/EBP β mRNA a slow, regulated polyadenylation of the message occurs. In our β d/s construct, the site is deleted, so polyadenylation would be expected to occur at an enhanced rate followed by translocation to the cytosol, leading to increased cytosolic message and protein relative to the cells expressing the wild type C/EBP β . We are currently testing this hypothesis.

Finally we suggest that this complex pathway that results in the movement of the message from the nucleus to the translational apparatus presents an ideal target for intervention in the onset of obesity.

Current Publications on the Project:

Gantt, K.R., Jain, R.G., Dudek, R., and **Pekala, P.H.** (2004) HuB localizes to polysomes and alters C/EBP- β expression in 3T3-L1 adipocytes. *Biochem. Biophys. Res. Commun.* 313: 619-622

Gantt, K., Cherry, J., Tenney, R., Karschner, V., and **Pekala, P.H.** (2005) An early event in adipogenesis: the nuclear selection of the C/EBP β mRNA by HuR and its translocation to the cytosol. *J. Biol. Chem.* 280: 24765-24774.

Cherry, J., Karschner, V., Jones, H., and **Pekala, P.H.** (2006) HuR, an RNA binding protein involved in cellular differentiation. *In Vivo* 20: 17-24.

Gantt, K., Cherry, J., Atasoy, U., Karschner, V., Richardson, M., and **Pekala, P. H.** (2006) Regulation of glucose transporter (GLUT1) expression by the RNA binding protein HuR. *J. Cell. Biochem.* 99: 565-574.

Jones, H., Carver, M., and **Pekala, P.H.** (2007) HuR binds to a single site on the C/EBP β mRNA of 3T3-L1 adipocytes. *Biochem. Biophys Res. Commun.* In press.

Cherry, J., Jones, H., Karschner, V., and **Pekala, P.H.** (2007) Expression of C/EBP β and the control of adipocyte differentiation: Influence of the C/EBP β 3'UTR and the binding of HuR. (2007). To be submitted – will be forwarded when accepted for publication.