In 3T3-L1 cells, HuR is constitutively expressed and prior to induction of differentiation localized predominantly to the nucleus. Within minutes of induction of differentiation, nuclear HuR binds to its target ligand mRNAs, and the complexes appear to move to the cytosol. One ligand mRNA is the CCAAT/enhancer-binding protein β (C/EBPβ) message. To examine the function and importance of the HuR-C/EBPβ interaction, retroviral expression constructs were created in which the HuR binding site was altered by deletion (βdel) or deletion and substitution (βd/s). Expression of these constructs in murine embryonic fibroblasts resulted in significant adipose conversion relative to those cells expressing wild type C/EBPβ. C/EBPβ protein content was increased markedly in both βdel and βd/s, which correlated with the acquisition of the adipocyte phenotype. Analysis of the βd/s cell line demonstrated a robust expression of C/EBPα coincident with peroxisome proliferator-activated receptor γ expression. Total C/EBPβ mRNA accumulation indicated no difference between cells harboring either the wild type C/EBPβ cDNA or βd/s construct. However, cytosolic C/EBPβ mRNA in the cells expressing the βd/s construct was maintained at levels between 2- and 7-fold greater than in the cells expressing the wild type construct. Alteration in mRNA half-life was not responsible for the increased accumulation. Mechanistically, these data suggest that HuR binding results in nuclear retention of the C/EBPβ mRNA and is consistent with HuR control, at least in part, of mRNA processing.

Adipocyte differentiation is a complex process regulated in large part by the temporally controlled expression and activation of numerous transcription factors (1). Among these proteins, the CCAAT/enhancer-binding protein β (C/EBPβ)3 and peroxisome proliferator-activated receptor (PPAR) families of transcriptional activators have been identified as critical to initiation of the differentiation process as well as to maintenance of the adipocyte phenotype (1). Functional roles for these factors have been established at least in part through use of the 3T3-L1 preadipocyte model system (1). When 3T3-L1 preadipocytes are induced to differentiate, the cells reenter the cell cycle and undergo mitotic clonal expansion followed by growth arrest and expression of the adipocyte phenotype (1). During this process, C/EBPβ is expressed coincidentally with induction of differentiation and is essential not only for mitotic clonal expansion but also for the transcriptional activation of PPARγ and C/EBPα genes (2–6). The indispensable nature of appropriate C/EBPβ expression was demonstrated in studies with C/EBPβ−/− murine embryonic fibroblasts (MEFs), which when treated with the differentiation inducers could neither reenter the cell cycle and undergo mitotic clonal expansion nor express the adipocyte phenotype (4). Similar results were observed in 3T3-L1 cells expressing a dominant-negative C/EBPβ (5).

Messenger RNA export from the nucleus, mRNA turnover, and 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. One of these proteins is HuR, a 36-kDa protein that belongs to the Hu/ELAV (embryonic lethal abnormal vision) family of RNA-binding proteins (7). HuR is ubiquitously expressed, localized predominantly to the nucleus, and has been demonstrated to shuttle between the nucleus and cytoplasm. The shuttling activity suggests but has not yet proved that HuR functions by binding to nascent mRNAs in the nucleus and protecting them from degradation by actively participating in their nucleocytoplasmic transport (8–23). Once in the cytosol, there is compelling evidence to suggest that HuR functions to control the stability and translational efficiency of its ligand mRNAs (8–26). Recent data have also supported a role for HuR in the regulation of polyadenylation by competitively inhibiting the binding of the cleavage and polyadenylation specificity factor, thereby attenuating polyadenylation and nuclear export (27). It is not clear that any one mRNA is subjected to all four HuR-mediated regulations, i.e. 1) control of polyadenylation, 2) translocation

PPAR, peroxisome proliferator-activated receptor; UTR, untranslated region; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; mRNP, messenger ribonucleoprotein.

Post-transcriptional Control of CCAAT/Enhancer-binding Protein β (C/EBPβ) Expression

FORMATION OF A NUCLEAR HuR-C/EBPβ mRNA COMPLEX DETERMINES THE AMOUNT OF MESSAGE REACHING THE CYTOSOL*

Joy Cherry, Heath Jones, Vesna A. Karschner, and Phillip H. Pekala*

From the Department of Biochemistry and Molecular Biology, The Brody School of Medicine at East Carolina University, Greenville, North Carolina 27858

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; MEF, murine embryonic fibroblast; UTP, uridine 5'-triphosphate; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VD, vitamin D; PPARγ, peroxisome proliferator-activated receptor γ; PPARα, peroxisome proliferator-activated receptor α; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatitis virus G protein; ARE, AU-rich element; EV, empty vector; VSV-G, vesicular stomatis...
to the cytosol, 3) stability, and 4) translational initiation/efficiency. It is important to realize that HuR is a regulatory protein involved in the post-transcriptional processing of certain mRNAs and that the particular function(s) may depend on the particular message.

In 3T3-L1 preadipocytes, HuR is constitutively expressed and localized predominantly to the nucleus (28). 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. We have demonstrated previously (28) that one of the relevant mRNAs forming a nuclear complex with HuR upon induction of the differentiation program and translocating to the cytosol as a messenger ribonucleoprotein (mRNP) complex with HuR is the C/EBPα message.

In this study, we begin to address the functional significance of the interactions between HuR and the C/EBPα 3′-untranslated region (UTR). Using non-adipogenic MEF-3T3 cells, we examined the effects of conditional ectopic expression of C/EBPα and mutants unable to bind HuR on the metabolism of the C/EBPα message.

**EXPERIMENTAL PROCEDURES**

Materials—Dulbecco’s modified Eagle’s medium (DMEM), Opti-MEM I reduced serum medium, RNase inhibitor, Lipofectamine 2000, and reagents for molecular biology were purchased from Invitrogen. The BD Retro-X Universal packaging system, the RevTet-Off™ system, the MEF/3T3 Tet-Off cell line, and the vesicular stomatitis virus G protein (VSV-G) and enhanced green fluorescent protein expression vectors were obtained from BD Biosciences/Clontech. Bovine calf serum and fetal calf serum were purchased from Hyclone Laboratories (Logan, UT). The 3T3-L1 cells used in this work were obtained from BD Biosciences/Clontech. Bovine calf serum and enhanced green fluorescent protein expression vectors were obtained from BD Biosciences/Clontech. Bovine calf serum and fetal calf serum were purchased from Hyclone Laboratories (Logan, UT). The 3T3-L1 cells used in this work were obtained from BD Biosciences/Clontech.

**Isolation of Nuclear and Cytosolic Fractions**—In 3T3-L1 preadipocytes, HuR is constitutively expressed and localized predominantly to the nucleus (28). 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. We have demonstrated previously (28) that one of the relevant mRNAs forming a nuclear complex with HuR upon induction of the differentiation program and translocating to the cytosol as a messenger ribonucleoprotein (mRNP) complex with HuR is the C/EBPα message.

In this study, we begin to address the functional significance of the interactions between HuR and the C/EBPα 3′-untranslated region (UTR). Using non-adipogenic MEF-3T3 cells, we examined the effects of conditional ectopic expression of C/EBPα and mutants unable to bind HuR on the metabolism of the C/EBPα message.

**EXPERIMENTAL PROCEDURES**

Materials—Dulbecco’s modified Eagle’s medium (DMEM), Opti-MEM I reduced serum medium, RNase inhibitor, Lipofectamine 2000, and reagents for molecular biology were purchased from Invitrogen. The BD Retro-X Universal packaging system, the RevTet-Off™ system, the MEF/3T3 Tet-Off cell line, and the vesicular stomatitis virus G protein (VSV-G) and enhanced green fluorescent protein expression vectors were obtained from BD Biosciences/Clontech. Bovine calf serum and fetal calf serum were purchased from Hyclone Laboratories (Logan, UT). The 3T3-L1 cells used in this work were obtained from BD Biosciences/Clontech.

**Isolation of Nuclear and Cytosolic Fractions**—In 3T3-L1 preadipocytes, HuR is constitutively expressed and localized predominantly to the nucleus (28). 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. We have demonstrated previously (28) that one of the relevant mRNAs forming a nuclear complex with HuR upon induction of the differentiation program and translocating to the cytosol as a messenger ribonucleoprotein (mRNP) complex with HuR is the C/EBPα message.

In this study, we begin to address the functional significance of the interactions between HuR and the C/EBPα 3′-untranslated region (UTR). Using non-adipogenic MEF-3T3 cells, we examined the effects of conditional ectopic expression of C/EBPα and mutants unable to bind HuR on the metabolism of the C/EBPα message.

**EXPERIMENTAL PROCEDURES**

Materials—Dulbecco’s modified Eagle’s medium (DMEM), Opti-MEM I reduced serum medium, RNase inhibitor, Lipofectamine 2000, and reagents for molecular biology were purchased from Invitrogen. The BD Retro-X Universal packaging system, the RevTet-Off™ system, the MEF/3T3 Tet-Off cell line, and the vesicular stomatitis virus G protein (VSV-G) and enhanced green fluorescent protein expression vectors were obtained from BD Biosciences/Clontech. Bovine calf serum and fetal calf serum were purchased from Hyclone Laboratories (Logan, UT). The 3T3-L1 cells used in this work were obtained from BD Biosciences/Clontech.
tion was continued for an additional 30 min at room temperature.

Cell Culture and Transfections—GP2-293 packaging cells were maintained in growth medium consisting of DMEM with 10% fetal bovine serum (FBS) on collagen I-coated plates. Upon reaching 90% confluence, the medium was changed to Opti-MEM I reduced serum medium with 10% FBS (no antibiotics). Co-transfections of the particular β construct along with an expression construct for VSV-G were performed utilizing Lipofectamine 2000 according to the manufacturer’s recommendations. All transfections were accompanied by an enhanced green fluorescent protein transfection efficiency control. At 6 h post-transfection, the medium was changed to fresh DMEM with 10% FBS. At 48 h after the medium change, the virus particle-containing supernatants were harvested, filtered through a 0.45-μm filter, and either used immediately or stored at −80 °C. A second harvest was carried out at 96 h. Production of virus particles was confirmed by Western blot analysis for VSV-G in the culture supernatants.

Transductions—The multipotential MEFs (MEF/3T3 Tet-Off cell line) express the tetracycline-controlled transactivator and were cultured in growth medium consisting of DMEM containing 10% calf serum and 100 ng/ml doxycycline on 6-well plates. Cells were plated and transduced at 15–20% confluence by addition of 2 ml of virus particle-containing medium. Plates were centrifuged at room temperature for 90 min at 2000 rpm, in effect pelleting the virus particles onto the cell membrane. Following an overnight incubation, the medium was changed to DMEM with 10% FBS and 10 ng/ml doxycycline. For cells that would follow the differentiation protocol, doxycycline was removed at 50% confluence. Cells were allowed to reach confluence, and then the medium was changed to DMEM supplemented with 10% FBS, 10 μg/ml insulin, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 10 μM troglitizone, with the control set of transductants receiving the 10 ng/ml doxycycline to repress transduced gene expression. At 48 h following the initial insulin/dexamethasone/3-isobutyl-1-methylxanthine treatment, the medium was replaced with DMEM containing 10% FBS, 10 μg/ml insulin, 10 μM troglitizone, and 10 ng/ml doxycycline where applicable. 48 h later, the medium was replaced with DMEM with 10% FBS, 2.5 μg/ml insulin, 10 μM troglitizone, and 10 ng/ml doxycycline where applicable. Notably, because of the high transduction efficiency (always in excess of 90%, similar to reports by Neal and Clipstone (29)), no drug selection was required. In every experiment, control transductions were performed in duplicate using a construct in which expression of enhanced green fluorescent protein was quantified by flow cytometry.

Real-time PCR—Real-time PCR analysis was performed essentially as we have described previously (30). Briefly, total RNA (0.5 μg, integrity demonstrated by ethidium-stained agarose gels) was subjected to reverse transcription with random primers and reverse transcriptase from the iScriptTM cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR was performed with specific primers designed for each gene with the Beacon Designer tool (Bio-Rad). Amplification and detection were done with the iCycler IQ real-time PCR detection system with IQ SYBR Green Supermix (Bio-Rad). Standard curves were prepared for each target gene, and PCR efficiency was determined to be in excess of 90% for all primer sets. Threshold temperatures were selected automatically, and all amplifications were followed by melt-curve analysis, i.e. plot of the negative first derivative of the fluorescence versus temperature with the software assigning the melt temperature. Single-melt temperatures were recorded in all cases. All primers were subjected to Blast search to ensure specificity and fold analysis to eliminate any primers with potential to form secondary structure. To calculate relative C/EBPβ mRNA, the threshold cycle (Ct) determined for the cells transduced with the empty vector (EV) (endogenous C/EBPβ) was subtracted from the average Ct for βwt and βΔs (ΔCt), thus correcting for the minor levels of endogenous expression. A standard curve was generated for each real-time PCR determination using a dilution series (50 ng, 33.3 ng, 11.1 ng, 3.7 ng, 367 pg, 120 pg) of total RNA from 3T3-L1 adipocytes (day 2). A plot of log starting quantity (ng) on the x axis and the Ct on the y axis was utilized to determine the arbitrary C/EBPβ mRNA levels of the unknown samples. Real-time PCR analyses with 90% or higher efficiency were utilized for quantification. For multiple independent runs after normalization to β-laminin expression, results were corrected for endogenous C/EBPβ expression using the Ct values obtained from the cells harboring the EV.

C/EBPβ mRNA Half-life Determination—The MEF-3T3 cells were transduced with the series of constructs and induced to differentiate as described above. At 24 h after induction of differentiation, doxycycline was added at a final concentration of 0.2 μM to the cultures to stop transcription. Total RNA was then isolated with respect to time, and analysis of C/EBPβ mRNA content was carried out using real-time PCR as described above. The data were plotted as log RNA content versus time. The equation y = ae−bt was fitted to the data, and half-lives were calculated.

Polyadenylation of the C/EBPβ mRNA—Polyadenylation of the C/EBPβ mRNA was determined using the primer/adapter reverse transcription-PCR method as described by Huarte et al. (31). The primer/adaptor utilized for reverse transcription was the following: 5′-d(T)12-GCGCCGGGCGTCGAGCAGC-3′. For the PCR, the forward primer was 5′-AAACCTTGCCTTGCAGGCGGTGTG-3′, and the reverse primer was the primer/adaptor described above.

RESULTS

Ectopic Expression of Both Wild Type and Mutant C/EBPβ mRNAs in the Multipotential Precursor MEF-3T3 Cells—Our previous work (28) demonstrated that upon 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. Our recent detailed analysis demonstrated the presence of a single binding site for HuR in the C/EBPβ mRNA. That site is in the AU-rich element (ARE) in the 3′-UTR of the message (32). Therefore, to address the function of HuR in the translocation and expression of C/EBPβ, we created constructs that expressed wild type C/EBPβ as well as mutants that could not bind HuR (Fig. 1A): 1) full-length wild type C/EBPβ cDNA (βwt), 2) C/EBPβ cDNA with point mutations at each end of the ARE (βΔp), 3) deletion of the ARE
HuR and C/EBPβ Expression

A. C/EBPβ Constructs

![Diagram](image)

(Bwt), and 4) deletion of the ARE and substitution with a sequence that did not contain a HuR binding site (βd/s).

Riboprobes (~150 bases in length) were prepared containing the ARE for the Bwt and βpm constructs; the probe for the βd/s construct contained the substituted region (Fig. 1A), whereas the probe for the Bdel construct contained 75 bases flanking either side of the deleted ARE. Gel shift assays were performed with adipocyte total cell lysates as a source of HuR protein to determine the ability of the probes to bind HuR. In Fig. 1B, lanes 1, 4, 7, and 10 display the various probes in the absence of added protein; lanes 2, 5, 8, and 11 show the probes + protein; and lanes 3, 6, 9, and 12 display the interaction of the probes with protein and HuR antibody. As demonstrated in lanes 1–3, the Bwt ARE forms a complex with HuR and is recognized by the HuR antibody, as we have demonstrated previously (28, 32). Lanes 4–6 display the βpm riboprobe in which single base changes at each terminus of the ARE were made, and, as shown in the figure, binding was not altered. However, when the ARE was deleted (Bdel; lanes 7–9) or deleted and substituted with a similar sized fragment (βd/s; lanes 10–12), HuR binding was lost. We do note that proteins other than HuR must bind to both Bdel and βd/s as judged by the loss of the probe band and appearance of complexes of higher mobility. There is minimal homology between the Bdel and βd/s probes; the sequence 66gctctgataacctg77 within βd/s exhibits 13 identical matches (boldface) within an 18-base region between positions 13 and 30 of the Bdel probe. We cannot address whether this homology is significant enough to be responsible for the complexes observed on the gel or if the complexes are the result of nonspecific binding. Our data do, however, support that these complexes do not involve HuR.

The constructs were then subcloned into the pRevTRE vector, a retroviral tetracycline/doxycycline-regulated expression system, RevTet-OffTM, and packaged by co-transfection with pVSV-G (encoding the vesicular stomatitis virus glycoprotein) into the GP2–293 pantropic packaging cell line using Lipofectamine® 2000. The virus particles produced were then used to transduce MEF-3T3 cells (see “Experimental Procedures”), which express HuR at normal levels but very low amounts of endogenous C/EBPβ. This approach is similar to that of Farmer and colleagues (33, 34), in which conditional ectopic expression of C/EBPβ in the multipotential NIH 3T3 cells in the presence of the differentiation induction mixture induced PPARγ and stimulated adipogenesis. Preliminary experiments confirmed that, similar to our previous studies with 3T3-L1 cells (28), the C/EBPβ mRNA was a ligand for HuR in the transduced MEF-3T3 cells.4

We then examined the effect of expression of the C/EBPβ constructs on the ability of the MEF-3T3 cells to differentiate. As shown in Fig. 2, based on oil red O staining at 8 days after induction of differentiation, expression of all four constructs led to the accumulation of triacylglycerol to some degree, with the greatest amount present in the Bdel and βd/s. This was unexpected as these two constructs, as demonstrated in Fig. 1B, do not bind HuR. The absence of triacylglycerol deposits in the cells maintained in doxycycline and those transduced with the EV supports the premise that expression of the various β constructs is responsible for the altered phenotype.

The expression level of C/EBPβ protein in the five transduced cell lines on day 8 after induction of differentiation is shown in Fig. 3. 25 µg of total cellular protein was analyzed by Western blotting, and full-length C/EBPβ (liver activating protein, 35- and 38-kDa species) was observed to be expressed significantly (greater than 3-fold) above endogenous (cells carried...
HuR and C/EBPβ Expression

| -doxycycline | +doxycycline |
|--------------|--------------|
| βwt | ![Image](image1.png) |
| βpm | ![Image](image2.png) |
| βdel | ![Image](image3.png) |
| βd/s | ![Image](image4.png) |
| EV | ![Image](image5.png) |

**FIGURE 2.** Expression of the adipocyte phenotype in MEF-3T3 cells transduced with expression constructs for wild type C/EBPβ and mutants that no longer bind HuR. MEF-3T3 cells were transduced with pRevTRE expression vector containing either the C/EBPβ cDNA or constructs altered at the HuR binding site (Fig. 1A). Expression of the constructs was up-regulated by removal of doxycycline from the culture medium, and the cells were induced to differentiate as described under “Experimental Procedures.” Cultures maintained in doxycycline resulted in suppression of construct expression and served as the negative controls. At day 8 post-induction of differentiation, the accumulated triacylglycerol in the cells was stained with oil red O. The data presented are representative of an experiment performed independently at least four times.

Carrying the EV) levels in all four cell lines transduced with a β construct. The cells carrying the βd/s and βdel constructs expressed both the 35- and 38-kDa forms of C/EBPβ at ~8-fold above EV and were the only cell lines in which the dominant-negative isoform liver inhibitory protein (20 kDa) was expressed at detectable levels. The mRNAs generated by the βd/s and βdel constructs did not bind HuR (Fig. 1B) yet expressed C/EBPβ protein at levels significantly above those found endogenously or in cells containing the βwt or βpm constructs. Thus, HuR binding to the ARE in the C/EBPβ message is not necessary for either movement of the message to the cytosol or expression of C/EBPβ protein. The enhanced expression driven by the βd/s and βdel constructs is consistent with HuR mediating an attenuation of expression when bound to the C/EBPβ ARE.

Further experimentation was carried out with only three of the constructs, βwt, βd/s, and EV. As the βd/s cell line accumulated more triacylglycerol, appearing to undergo a more thorough differentiation, and expressed more C/EBPβ protein, we next examined other markers of differentiation. The Western blot analysis for C/EBPα and PPARγ displayed in Fig. 4 indicates that increased expression of C/EBPβ in the βd/s cells resulted in increased expression of C/EBPα relative to either the βwt or EV cell lines. PPARγ levels were found to be identical in both βwt and βd/s, suggesting that even the low levels of C/EBPβ found in the βwt cells was sufficient to drive maximal expression of PPARγ.

**Accumulation of C/EBPβ mRNA in βwt and βd/s Cell Lines**—We examined the accumulation of total cellular C/EBPβ mRNA after removal of doxycycline and exposure of the cells to the differentiation mixture. The results shown in Fig. 5A indicate that total mRNA in both βwt and βd/s cells accumulated in a similar manner. The loss of the HuR-binding ARE in βd/s had no effect on the accumulation of total cellular message. This might be expected as the constructs were driven by the same tetracycline/doxycycline-regulated promoter.

We next examined the appearance and accumulation of C/EBPβ mRNA in the cytosolic compartment. Using the Pierce NE-PE RT™ kit, we isolated cytosolic and nuclear compartments prior to and after removal of doxycycline and induction of differentiation, as we have described previously (28). The real-time PCR data shown in Fig. 5B indicate that at all time points βd/s mRNA accumulated in the cytosol to a greater degree than the βwt message. Thus, the loss of the ability to bind HuR at the canonical ARE (present in βwt, but absent in βd/s) did not hinder the movement of the C/EBPβ mRNA into the cytosol. The previous data (Fig. 5A) indicated that total cellular C/EBPβ mRNA accumulated to a similar degree in both βwt and βd/s. These data (Fig. 5B) would suggest that a greater percentage of the total βd/s mRNA is in the cytosol, available for translation and driving the accumulation of C/EBPβ protein shown in Figs. 3 and 4. To confirm this hypothesis, we selected the 48-h time point and performed five independent isolations and subsequent determinations of the cytosolic versus nuclear distribution of βwt and βd/s. Those data are displayed in Fig. 5C and demonstrate that there is approximately two the βd/s mRNA in the cytosol relative to the βwt mRNA. We note that in separate experiments a minimum 2-fold differential has been
half-lives of 120 and 60 min, respectively, calculated using exponential decay regression. With the consideration that the \( \beta d/s \) mRNA has a more rapid half-life, the increased cytosolic accumulation is all the more significant.

\textit{C/EBP\( \beta \) mRNA Polyadenylation}—The data presented to this point are consistent with involvement of HuR in nuclear processing of the C/EBP\( \beta \) message. It would appear that once this mRNP complex forms, its translocation to the cytosol is attenuated. To investigate the influence of HuR on nuclear processing of the C/EBP\( \beta \) mRNA, we examined nuclear polyadenylation of the C/EBP\( \beta \) transcripts from the \( \beta wt \) and \( \beta d/s \) cell lines. To accumulate the data presented in Fig. 7, RNA was isolated from the nuclear fraction and subjected to reverse transcription using an oligo(dT) primer/adapter followed by PCR using a forward primer located 298 nucleotides upstream of the site of poly(A) addition of the C/EBP\( \beta \) mRNA in conjunction with the \( ^{32}P \)-labeled oligo(dT) primer/adapter. Reverse transcription-PCR with these primers of the mRNA from the \( \beta d/s \) cells (Fig. 7, lane 4) produced a smear of products ranging in size from \( \sim 300 \) to almost 400 nucleotides. The minimal size predicted was 329 nucleotides (298 bp of C/EBP\( \beta \) plus 31 nucleotides of the primer/adapter). Whereas there is evidence of polyadenylation with \( \beta wt \) (Fig. 7, lane 2), densitometric analysis indicated that it is \( \sim 35\% \) that found in the \( \beta d/s \). Notably, there is no evidence of polyadenylation occurring when mRNA was isolated from cells containing the empty vector (Fig. 7, lane 6). The data suggest that in the absence of HuR binding, the C/EBP\( \beta \) mRNA is more extensively polyadenylated, leading to translocation to the cytosol. However, in \( \beta wt \), which binds HuR, polyadenylation appears to occur to a lesser extent, with approximately one-third of the RNA (relative to \( \beta d/s \) reaching the cytosol. Similar results were obtained using an RNase H-based approach for determination of poly(A) tail size for total cellular RNA.\(^5\)

\textbf{DISCUSSION}

As preadipocytes differentiate, controlled expression of C/EBP\( \beta \) is essential to acquisition of the adipose phenotype. Transcriptional activation of the C/EBP\( \beta \) gene in 3T3-L1 cells occurs within minutes of exposure to the differentiation inducers and is controlled, at least in part, by the cAMP-response element-binding protein (35, 36). In the differentiation program of 3T3-L1 cells, C/EBP\( \beta \) first controls the entry of the cells into mitotic clonal expansion, and then the expression of C/EBP\( \alpha \) and PPAR\( \gamma \) (2–6, 33, 34). The timing of expression during these processes is critical because C/EBP\( \beta \) is promitotic and C/EBP\( \alpha \) is antimitotic, and thus C/EBP\( \beta \) expression must attenuate as C/EBP\( \alpha \) expression initiates. This study describes a critical early post-transcriptional regulation initiated in the nucleus involving formation of a HuR-C/EBP\( \beta \) mRNA complex. Formation of this mRNP appears to control the rate of C/EBP\( \beta \) mRNA translocation to the cytosol but is not essential for the translocation process itself. In our previous work, we identified the HuR binding site in the C/EBP\( \beta \) 3‘-UTR and demonstrated that it is the only site within the entire message (32). As evidenced in the data presented in Fig. 5, deletion of this

\(^5\) V. A. Karschner and P. H. Pekala, unpublished data.

\textbf{Figure 4.} C/EBP\( \alpha \) is expressed to a greater degree in the \( \beta d/s \) cells. At 8 days post-induction of differentiation, cell extracts were prepared, and 25 \( \mu \)g of protein of the three cell lines indicated was subjected to Western blot analysis for C/EBP\( \alpha \), C/EBP\( \beta \), and PPAR\( \gamma \). Molecular masses are indicated in kDa. B–D are from the same plot probed sequentially for C/EBP\( \beta \), PPAR\( \gamma \), and \( \beta \)-tubulin. A is from a separate Western blot. The experiment was performed twice with identical results.
HuR binding site did not disrupt nuclear to cytoplasmic translocation of the message. Indeed, in the absence of HuR binding, more C/EBP mRNA was localized to the cytosol. Conceivably, accumulation of d/s in the cytosol could be a consequence of deletion of the ARE instability element, resulting in a stabilized message. However, as displayed in Fig. 6, the d/s mRNA actually has a shorter half-life than the wt message, making its accumulation more difficult. Overall, our data are consistent with C/EBP mRNA translocation to the cytosol occurring more readily when HuR is not bound, consistent with HuR functioning as a “brake” or attenuator of movement of the complex to the cytosol. When HuR binds to the C/EBP message in the nucleus, movement to the cytosol is not prohibited, simply diminished. The C/EBP mRNA reaches the cytosol in lower three times with similar results. Data are plotted as the mean ± S.D. C, the levels of wt and d/s mRNAs in the cytosol at the 48-h time point (five independent determinations) are shown. Normalization was to β-laminin mRNA, the expression of which does not change over the time course of the experiment, followed by correction for endogenous expression as discussed under “Experimental Procedures.” wt content was defined as 100%, and d/s is expressed relative to that value.
HuR and C/EBPβ Expression

The apparent disparity between the levels of increased βd/s mRNA and C/EBPβ protein levels has led us to focus on the involvement of HuR in the control of translational efficiency, and those studies are currently in progress.

Our current data describe the involvement of HuR in the metabolism of the C/EBPβ message and indicate that formation of the mRNP particle in the nucleus controls the quantity of message entering the cytosol. On the same time frame, HuR binds to other ligand mRNAs that may also play critical roles in the differentiation process. For example, β-actin can be found in both nuclear and cytosolic complexes with HuR 30 min after induction of differentiation.2 The function of that interaction remains unknown. Although it was not the goal of this study, the data presented demonstrate very effectively that the interaction between HuR and C/EBPβ mRNA is not necessary for initiation of the differentiation program. Thus, our previously described attenuation of the differentiation process by small interfering RNA-mediated suppression of HuR was not a consequence of the loss of interaction with C/EBPβ mRNA. Other ligand mRNAs that are dependent on interaction with HuR to perhaps stabilize the message or increase its translational efficiency to produce a functional protein must be involved. In attempting to identify these critical mRNAs, we are focusing on the immediate early gene mRNAs, including c-fos, Krox20, fosB, and c-jun, all of which have apparent HuR binding sites. At least c-fos and c-jun have been demonstrated to be ligands for HuR in other systems (16). Moreover, we have previously documented activation of the transient expression of these immediate early genes within minutes of exposure to the differentiation mixture (39), making them potentially ideal targets for HuR binding.

Finally, it is interesting that genetic approaches have identified mouse strains with mutations in the HuR binding motif of inflammatory mRNAs that correlate with the development of autoimmunity (40). On the basis of these observations, we suggest that, at least in some cases, alterations in the ability of HuR to bind the C/EBPβ mRNA could lead to overexpression of C/EBPβ protein and result in the onset of adipogenesis and potentially the disease state of obesity. This we believe merits investigation.

Acknowledgments—We gratefully acknowledge the expert laboratory support of Melinda Carver, Becky Keener, Ashley Ferguson, and Mitch Harris. We also thank Dr. Joseph M. Chalovich for aid in determination of the mRNA half-lives and Drs. Kira Gantt and Tania Kastelic for thoughtful criticism on reading the manuscript.

REFERENCES

1. Otto, T. C., and Lane, M. D. (2005) Mol. Biol. 40, 229–242
2. Zuo, Y., Qiang, L., and Farmer, S. R. (2006) J. Biol. Chem. 281, 7960–7967
3. Hamm, J. K., Park, B. H., and Farmer, S. R. (2001) J. Biol. Chem. 276, 18464–18471
4. Tang, Q.-Q., Otto, T. C., and Lane, M. D. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 850–855
5. Zhang, J. W., Tang, Q.-Q., Vinson, C., and Lane, M. D. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 43–47
6. Zhang, J. W., Klemm, D. J., Vinson, C., and Lane, M. D. (2004) J. Biol. Chem. 279, 4471–4478
7. Robinow, S., Campos, A. R., Yao, K. M., and White, K. (1988) Science 242, 1570–1572

quantities and is translated into protein. The differentiation program is initiated and maintained. When the binding site is altered such that HuR cannot bind, more C/EBPβ mRNA per unit time is found in the cytosol. The presence of more message drives the overexpression of C/EBPβ protein, resulting in overexpression of C/EBPα protein and a more robust differentiation program.

Until recently, potential nuclear functions of the Hu proteins have not been addressed. However, several recent reports have supported roles for HuR as well as the neuronal specific Hu proteins in the nuclear processing of mRNAs with respect to the regulation of splicing as well as polyadenylation (27, 37, 38). The C/EBPβ message is derived from an intronless gene, thus, regulation at the level of splicing is not an option. However, the HuR binding site is in close proximity to the polyadenylation signal and is flanked by uridylate-rich regions. The sequence flanking the HuR binding site in the C/EBPβ mRNA is similar to that previously described for the non-neuronal alternative 3′-terminal exon 4 of the calcitonin/calcitonin gene-related peptide message. On that message, HuR was demonstrated to inhibit both cleavage and polyadenylation by competing for binding to the RNA with subunits of the cleavage and polyadenylation specificity factor. Our data are consistent with HuR serving this same function when bound to the C/EBPβ mRNA. This potential mechanism is currently under investigation.

Whereas these results specifically define a nuclear function for HuR in the metabolism of the C/EBPβ message, our previous data describing cytosolic HuR-C/EBPβ mRNP particles suggest that HuR functions in both compartments. HuR may be involved in other aspects of regulating the expression of
8. Fan, X. C., and Steitz, J. A. (1998) EMBO J. 17, 3448–3460
9. Myer, V. E., Fan, X. C., and Steitz, J. A. (1997) EMBO J. 16, 2130–2139
10. Levy, N. S., Chung, S., Furneaux, H., and Levy, A. P. (1998) J. Biol. Chem. 273, 6417–6420
11. Ma, W. J., Cheng, C. Y., Xu, N., and Shyu, A. B. (1996) Mol. Cell. Biol. 17, 5105–5114
12. Peng, S. S., Chen, C. Y., Xu, N., and Shyu, A. B. (1998) EMBO J. 17, 3461–3470
13. King, P. H., Fuller, J. J., Nabors, L. B., and Detloff P. J. (2000) Gene (Amst.) 242, 125–131
14. Ford, L. P., Watson, J., Keene, J. D., and Wilusz, J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1134–1143
15. Gallouzi, I. E., Brennan, C. M., Stenberg, M. G., Swanson, M. S., Eversole, A., Maizels, N., and Steitz, J. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3073–3078
16. Keene, J. D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5–7
17. Dixon, D. A., Tolley, N. D., King, P. H., Nabors, L. B., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (2001) J. Clin. Investig. 108, 1657–1665
18. Goldberg-Cohen, I., Furneaux, H., and Levy, A. P. (2002) J. Biol. Chem. 277, 13635–13640
19. Loflin, P., and Lever, J.E. (2001) FERS Lett. 509, 267–271
20. Dean, J. L., Wait, R., Mahtani, K. R., Sully, G., Clark, A. R., and Saklatvala, J. (2001) Mol. Cell. Biol. 21, 721–730
21. Kirigiti, P., Bai, Y., Yang, Y. F., Li, X., Li, B., Brewer, G., and Machida, C. A. (2001) Mol. Pharmacol. 60, 1308–1324
22. Brennan, C. M., Gallouzi, I. E., and Steitz, J. A. (2000) J. Cell Biol. 151, 1–13
23. Gallouzi, I. E., and Steitz, J. A. (2001) Science 294, 1895–1901
24. Jain, R. G., Andrews, L. G., McGowan, K. M., Pekala, P. H., and Keene, J. D. (1997) Mol. Cell. Biol. 7, 954–962
25. Gantt, K. R., Jain, R. G., Dudek, R. W., and Pekala, P. H. (2004) Biochim. Biophys. Res. Commun. 313, 619–622
26. Antic, D., Lu, N., and Keene, J. D. (1999) Genes Dev. 13, 449–461
27. Zhu, H., Zhou, H.-L., Hasman, R. A., and Lou, H. (2007) J. Biol. Chem. 282, 2203–2210
28. Gantt, K., Cherry, J., Tenney, R., Karschner, V., and Pekala, P. H. (2005) J. Biol. Chem. 280, 24765–24774
29. Neal, J. W., and Clipstone, N. A. (2003) J. Biol. Chem. 278, 17246–17254
30. Morrison, R., and Farmer, S. R. (1999) J. Biol. Chem. 274, 17088–17097
31. Morrison, R., and Farmer, S. R. (1999) J. Biol. Chem. 274, 17088–17097
32. Jones, H., Carver, M., and Pekala, P. H. (2007) Biochem. Biophys. Res. Commun. 355, 217–220
33. Wu, Z., Xie, Y., Morrison, R. F., Bucher, N., and Farmer, S. R. (1998) J. Clin. Investig. 101, 22–32
34. Morrison, R., and Farmer, S. R. (1999) J. Biol. Chem. 274, 17088–17097
35. Gonzalez, G. A., and Montminy, M. R. (1986) Cell 59, 675–680
36. Klemm, D. J., Roesler, W. J., Boras, T., Colton, L. A., Felder, K., and Reusch, J. E. (1998) J. Biol. Chem. 273, 917–923
37. Zhu, H., Hasman, R. A., Barron, V. A., Luo, G., and Lou, H. (2006) Mol. Biol. Cell 17, 5105–5114
38. Zhu, H., Himman, M. N., Hasman, R. A., Mehta, P., and Lou, H. (2008) Mol. Cell. Biol. 28, 1240–1251
39. Stephens, J. M., Butts, M., Stone, R., Pekala, P. H., and Bernlohr, D. A. (1999) Mol. Cell. Biochem. 123, 63–71
40. DiMarco, S., Hel, Z., Lachance, C., Furneaux, H., and Radzioch, D. (2001) Nucleic Acids Res. 29, 863–871