THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 26, Issue of July 1, pp. 24768–24774, 2005

Printed in U.S.A.

An Early Event in Adipogenesis, the Nuclear Selection of the CCAAT Enhancer-binding Protein β (C/EBPβ) mRNA by HuR and its Translocation to the Cytosol*

Received for publication, February 22, 2005, and in revised form, April 14, 2005
Published, JBC Papers in Press, April 29, 2005, DOI 10.1074/jbc.M502011200

Kira Gantt, Joy Cherry, Raleigh Tenney, Vesna 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 27858-4354

HuR is a ligand for nuclear mRNAs containing adenylate-uridylate-rich elements in the 3′-untranslated region. Once bound to the mRNA, HuR is recognized by adapter proteins that then facilitate nuclear export of the complex. In the cytosol, HuR is thought to function to control stability and translation of 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. Using *in vitro* RNA gel shifts, we have demonstrated that the CCAAT enhancer-binding protein β (C/EBPβ) message is a ligand for HuR. Within 2 h of initiation of the differentiation process, HuR complexes containing C/EBPβ mRNA could be isolated from the cytosolic compartment. Importantly, the process appears to be highly selective, as cyclin D1, which contains a putative HuR binding site and is expressed on the same time frame as C/EBPβ, was not found in the immunoprecipitated messenger ribonucleoprotein complexes. The proximity of this event to adipogenic stimuli and the importance of C/EBPβ to the differentiation process have led us to hypothesize a role for HuR in the regulation of the onset of adipogenesis. In support of this hypothesis, small interfering RNA suppression of HuR protein content resulted in an inhibition of C/EBPβ protein expression and an attenuation of the differentiation process.

When confluent 3T3-L1 preadipocytes are treated with differentiation inducers, they synchronously reenter the cell cycle and undergo approximately two rounds of cell division as they enter a process that has become known as mitotic clonal expansion (1–3). This increase in cell number and commitment to differentiation mimics the increase in adipose mass in obesity (4–6). In the 3T3-L1 preadipocytes, mitotic clonal expansion has been demonstrated to be required for terminal adipocyte differentiation (1, 2), and a transcription factor expressed prior to the start of the process, C/EBPβ,[1] plays an essential role in the mitotic clonal expansion as well as in subsequent events in the differentiation program (7–9). Transcription of the *C/EBPβ* gene is increased shortly after exposure of the cells to the differentiation inducers, and the C/EBPβ protein can be detected in the nucleus within 4 h (10). On termination of the mitotic clonal expansion phase, C/EBPβ has been proposed to be responsible for the transactivation of *C/EBPα* and *PPARγ* genes, two transcription factors responsible for establishing and maintaining the adipocyte phenotype (11–16). As C/EBPα expression is increased, C/EBPβ expression is attenuated (17). Thus, C/EBPβ plays a critical early regulatory role in the differentiation process.

HuR is an RNA-binding protein belonging to the Hu/ELAV family of mRNA-binding proteins and is expressed in adipose (this article), intestine, spleen, thymus, and testis with minor expression in liver and uterus (18, 19). HuR contains a nucleocytoplasmic shuttling sequence and functions as an adapter protein in the nuclear export of mRNAs that contain adenylate-uridylate-rich elements (AREs) in their 3′-untranslated regions (19–22, 24). Depending on the specific message, the nuclear HuR-containing messenger ribonucleoprotein (mRNP) is bound by APRIL and/or pp32 and then CRM1, which is recognized by a specific binding domain on 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 (25, 26). Once in the cytosol, HuR functions to stabilize mRNA ligands (27, 28). Based on results obtained with the ectopic expression of the neuronal Hu family homolog HuB in both the 3T3-L1 adipocytes and human teratocarcinoma cells, HuR may also participate in control of translation initiation (29, 30). The selection of ligands and the translocation of HuR to the cytosol has been proposed to be under tight control (31). 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 coactivator-associated arginine methyltransferase (CARM) appears to control the process (31).

HuR has been suggested to be involved in control of the cell cycle through stabilization of the mRNAs for cyclins A and B1 during S and G_2, leading to increased expression of these proteins (32). HuR involvement in differentiation was supported by RNA interference knockdowns of the protein in C2C12 cells, which resulted in an inhibition of myogenesis coincident with decreased

* The authors gratefully acknowledge the support of National Institutes of Health Grant DK55169 and American Diabetes Association Research Grant 7-03-RA-76 in the performance of these studies. 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.

‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, The Brody School of Medicine at East Carolina University, 600 Moye Blvd., Greenville, NC 27858-4354. Tel.: 252-744-2684; Fax: 252-744-3393; E-mail: pekalap@mail.ecu.edu.

[1] The abbreviations used are: C/EBPβ, CCAAT enhancer-binding protein; ARE, adenylate-uridylate-rich element; mRNP, messenger ribonucleoprotein; PPAR, peroxisome proliferator-activated receptor; Sc, Dharmacon siCONTROL non-targeting siRNA™ four-oligo pool; siRNA, small interfering RNA; UTR, untranslated region.

expression of MyoD and myogenin mRNAs, known ligands for HuR (33). Thus, it might 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. In the current study we demonstrate that the C/EBPβ message is a ligand for HuR and that the time frame for cytosolic translocation of the complex is consistent with an early regulatory event in the differentiation process.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium was purchased from Invitrogen. Bovine calf serum and fetal calf serum were purchased from Hyclone Laboratories (Logan, Utah). The 3T3-L1 cells used in this work were obtained from Howard Green (Harvard University, Boston, MA). The BCA protein assay kit, the NE-PER™ cell fractionation kit, and the HALT™ protease inhibitor mix were from Pierce. The RNAqueous RNA extraction kit was from Ambion (Austin, TX). Reagents for molecular biology were purchased from Invitrogen. All other chemicals were of reagent grade and were purchased from Sigma-Aldrich. The 3A2 monoclonal antibody directed against HuR and the β-tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) The mouse anti-Armenian and anti-Syrian hamster IgG1 monoclonal antibody used as an isotype control was obtained from BD Biosciences. Antibodies directed against C/EBPβ were provided by M. Daniel Lane (The Johns Hopkins University, Baltimore, MD). The antibody against HnRNP70 was provided by Jack Keene (Duke University, Durham, NC). The siGENOME SMARTpool™ reagent and the siCONTROL™ non-targeting siRNA were obtained from Dharmacon (Lafayette, CO),

Primers—The primers used for PCR were designed using the GCG program Prime. All sequences determined were subjected to a BLAST search to ensure selectivity of hybridization. These primers and their sequences were as follows: C/EBPβ, 5′-GCGGGGTTGTTGATGTTT-3′ (forward) and 5′-CTTTAATGCTCGAAACGG-3 (reverse); cyclin D1, 5′-GCTTGACCTTTCCCAACCC-3′ and 5′-TCACCTCTTCCCTCACATCC-3′ (reverse); and GATA3, 5′-AGTGTGCGAAGAGTTCCTCC-3′ (forward) and 5-TTTTTCACAGCACTAGAGACCC-3 (reverse). The T7 polymerase binding site was included in the double-stranded templates used in the preparation of riboprobes for RNA gel shift reactions by including the sequence 5′-GGAT CCTAATACGACTCACTATAGGGAGCT-3′ at the 5′-end of a forward primer.

3T3-L1 Cell Culture—3T3-L1 preadipocytes were cultured, maintained, and differentiated as described previously (29). For experimentation, preadipocytes were harvested at 2 days post-confluence, and adipocytes were used at 8 days post-induction of differentiation. Differentiation was induced by exposure of the cells to isobutyl methyl xanthine, dexamethasone, and insulin in the presence of 10% fetal bovine serum. These additions will be referred to as the "differentiation inducers" throughout the text.

Isolation of Cytosolic and Nuclear Fractions—The NE-PER™ cell fractionation kit was used to isolate cytosolic and nuclear fractions from the 3T3-L1 cells as per the manufacturer's (Pierce) instructions with minor modifications. Briefly, 5–10-cm culture dishes were used as a source of material for the separation. In addition to the standard protease inhibitors, an RNase inhibitor was added to the lysate. Incubation time for the initial extract was increased from 10 to 15 min. Once the cytosolic fraction was isolated, the nuclear fraction was subjected to a brief centrifugation, and the interface was removed to reduce cytoplasmic contamination. The isolated fractions were stored at −80C° until use. Because of high salt concentrations, nuclear fractions were dialyzed prior to immunoprecipitation.

Immunoprecipitation of mRNP Complexes—The mRNP complexes from either nuclear or cytosolic fractions were immunoprecipitated and mRNA isolated using RNase- and DNase-free conditions by a modification of the protocol described by Tennenbaum *et al.* (34). Protein A-Sepharose beads were resuspended overnight at 4 °C in 10 ml of HNTM buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, and 1% Triton X-100). For immunoprecipitation, 200 μg of cytosolic lysate or 100 μg of nuclear lysate was utilized. Four samples were prepared for each immunoprecipitation, three for the isolation of RNA and one as a Western blot analysis control. Starting with the protein extract in HNTM buffer, the following were added to an RNase/DNase-free microfuge tube: Halt protease inhibitor (10 μl), vanadyl ribonucleoside complex (10 μl), 40 units/μl RNase inhibitor (10 μl), 0.1 M dithiothreitol (1 μl), and 0.5 M EDTA (33 μl). The antibody was then added; for the isotype control, anti-Armenian/anti-Syrian hamster IgG1 monoclonal antibody was used, and, for HuR, monoclonal 3A2 was used. Finally, the appropriate volume of lysate was added and the mixture rotated for 3 h at room temperature followed by the addition of protein A-Sepharose beads with continued mixing for an additional 2 h at room temperature. This was followed by two washes with HNTM buffer alone. The mixture was then centrifuged (13,200 rpm at 4 °C) and washed three times with cold 1 M urea in HNTM buffer. One of the samples was taken at this time for the Western analysis control, and RNA was extracted from the remainder using the Ambion RNAqueous kit as per the manufacturer's instructions. Extracted RNA was stored at −80 °C.

Western Blot Analysis—Western blot analysis was performed as detailed previously (36). Because of the disparity in nuclear *versus* cytosolic HuR concentrations, 5 μg of nuclear protein and 25 μg of cytosolic protein were used for Western blot analysis.

RNA Gel Shift Analysis—Radiolabeled probes were prepared from double-stranded constructs containing the T7 polymerase promoter using the Ambion RNA probe synthesis and removal kit as per the manufacturer's instructions. The probe was gel-purified and used for gel shift assays at ~100,000 cpm of probe per assay. Approximately 2 μg of either cytosolic or nuclear protein was incubated with the probe in the presence of binding buffer (10 mM Hepes, pH 7.6, 3 mM $MgCl_2$, 40 mM KCl, 5% glycerol, and 1 mM dithiothreitol) for 30–60 min. If supershifts were being carried out, the antibody (1 μl) was added at this time and the binding reaction was continued for an additional 30 min at room temperature. At the end of the binding reaction heparin was added to a final concentration of 1 mg/ml, and the incubation was continued for a final 10 min at room temperature. An equal volume of 2× native gel sample buffer (100 mM Tris-HCl, 10% glycerol, and 0.0025% bromphenol blue, pH 8.6) was added to the reaction mixture. The gels were pre-run for 30 min at 250 volts, and then the sample was loaded and complexes were formed separated on a native 4% acrylamide gel in 1× Tris borate-EDTA. Gels were dried under vacuum and exposed to x-ray film.

siRNA Treatment of the 3T3-L1 Preadipocytes—Transfection of the cells was performed using Dharmacon siGENOME SMARTpool™ reagent (catalog number M-053812-00-0020), a four-siRNA oligo system designed specifically for HuR (mouse ELAV1; GenBank™ accession number NM_010485) by Dharmacon. Briefly, preadipocytes in 12-well plates at ~80% confluency were transfected with siRNA using Lipofectamine 2000 as a carrier according to manufacturer's instructions. Two control transfections were carried out, one with Lipofectamine 2000 alone and the other with Dharmacon siCONTROL non-targeting siRNA™ four-oligo pool (Sc) (catalog number D-001206-13-05). The cells were exposed to the transfection mixture for 6 h, at which time the transfection medium was replaced with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Twenty-four hours after removal of the transfection media, two monolayers of each treatment group were trypsinized and combined to generate an immediately confluent monolayer, essential for differentiation. Eighteen hours later (48 h after the initial transfection), the cells were exposed to the differentiation protocol. Cells were then analyzed for acquisition of the adipocyte phenotype.

RESULTS

HuR Is Constitutively Expressed in the 3T3-L1 Cells—The results displayed in Fig. 1*A* demonstrate the constitutive expression of HuR in 2-day post-preadipocytes and fully differentiated (day 8) adipocytes as well as the effectiveness of the Pierce NE-PER™ kit for cytosol *versus* nuclear separation. For this analysis, 5 μg of nuclear and 25 μg of cytosolic extract were separated, blotted, and probed with antibodies directed against HuR, HnRNP70, and β-tubulin. The later two were used as markers for nuclear and cytosolic compartments. It should be noted that the decrease in β-tubulin with differentiation (seen in Fig. 1*A*) has been reported previously (37).

In Fig. 1*B*, 10 μg of protein were utilized for the analysis of both compartments. The data demonstrate that HuR is expressed in the preadipocytes with expression increasing ~3-fold as the cells differentiate. Notable is the increased presence of HuR in the cytosol with respect to differentiation. Analysis of three separate experiments indicates that in the preadipocytes ~15 ± 5% of the total HuR resides in the cytosol, whereas in the fully differentiated adipocyte cytosolic HuR represents 40 ± 8% of total cellular HuR. We note that because we are comparing 2-day post-confluent (day 0) preadipocytes with

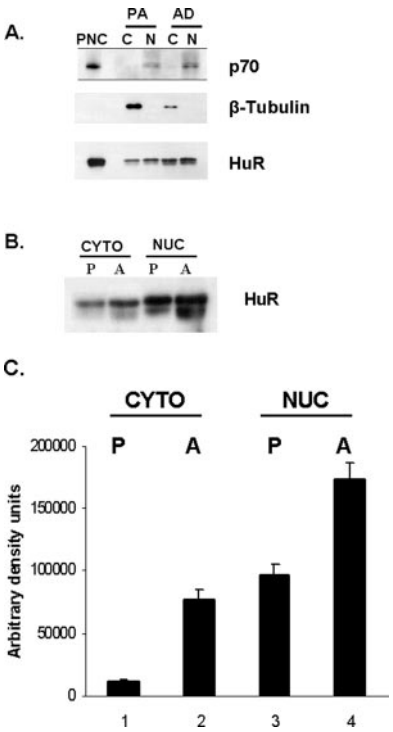

FIG. 1. **HuR is constitutively expressed in the 3T3-L1 cells.** Cytosolic and nuclear extracts were isolated from 2-day post-confluent preadipocytes (day 0) and fully differentiated adipocytes (day 8) using the Pierce NE-PER™ kit as per the manufacturer's instructions. *Panel A*, Western blot analysis was performed (25 μg of cytosolic extract and 5 μg of nuclear extract, based on HuR content for clarity of display) as described under "Experimental Procedures." The blot was first probed for HuR and then with anti-hnRNP 70 as a nuclear (*N*) marker and anti-β-tubulin as a cytosolic (*C*) marker. *PA*, preadipocyte; *AD*, adipocyte; *PNC*, positive nuclear control. *Panel B*, equal protein (10 μg) Western blot analysis of samples from *panel A*. *CYTO*, cytosolic; *NUC*, nuclear; *P*, preadipocyte; *A*, adipocyte. *Panel C*, quantification of HuR levels in cytosolic and nuclear compartments obtained using a Kodak Gel Logic 100 imaging system. Abbreviations are the same as in *panel B*. The data represent three separations.

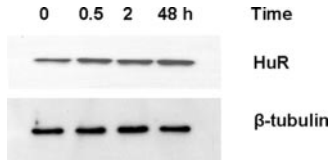

FIG. 2. **HuR moves to the cytosol soon after exposure of the cells to the differentiation inducers.** The time course of HuR movement to the cytosol by Western analysis is presented. Shown are samples of cytosolic extracts from time (*0*) prior to treatment of the preadipocytes with 3-isobutyl-1-methylxanthine, dexamethasone, and insulin to induce differentiation and 48 h after initiation of that treatment. The data shown are representative of three determinations of the time course of HuR translocation to the cytosol.

fully differentiated adipocytes in Fig. 1*B*, no marker protein is utilized for normalization. The protein content of the markers changes significantly during the differentiation process.

We then determined the time frame for HuR movement to the cytosol. Cytosolic fractions were prepared and assayed by Western blot analysis for the presence of the HuR protein with respect to the time after exposure of the preadipocytes to the differentiation inducers. As shown in Fig. 2, in the preadipocytes (2 days post-confluence, day 0/time 0) HuR is detectable in the cytosol. However, within 30 min after initiation of differentiation, the cytosolic content increased by 30%. Over the next 48 h the cytosolic content of HuR attained a maximum 45% increase over the preadipocyte content. (quantification of HuR movement was performed using a Kodak Gel Logic 100 imaging system and normalized to the β-tubulin levels.) The increase was maintained through day 8, when the cells were considered to be fully differentiated (data not shown). Thus, HuR exits the nucleus early and establishes a new equilibrium distribution between the nucleus and the cytosol and maintains that distribution in the terminally differentiated adipocyte. Note that the cytosolic marker β-tubulin, the concentration of which does not change during the time frame of the experiment, demonstrates even loading of the samples.

The C/EBPβ mRNA Is a Ligand for HuR in Vitro—The rapid translocation of HuR to the cytosol after the addition of differentiation inducers to the culture suggested that it might be involved in a critical early event in the differentiation process. Focusing on the role of HuR in the movement of specific mRNAs from the nucleus to the cytosol, we began to look for potential mRNA ligands.

Cole *et al.* (10) demonstrated that the C/EBPβ protein can be found in the nucleus within 4 h of the addition of the differentiation inducers to the 3T3-L1 preadipocytes. This would suggest that the C/EBPβ message must exit the nucleus shortly after the addition of the differentiation inducers. If formation of a HuR complex with the C/EBPβ message is obligatory for translocation to the cytosol, both HuR and C/EBPβ mRNA should be observed moving to the cytosol rapidly after the addition of the inducers.

Examination of the C/EBPβ sequence demonstrates the presence of an ARE in the 3′-UTR (Fig. 3*A*) that might serve as a potential binding site for HuR. Cytosolic extracts were prepared from fully differentiated adipocytes, and the RNA gel shift assays performed using a radiolabeled probe corresponding to the ARE are shown in Fig. 3*A*. As shown in Fig. 3*B*, *lane 2*, in the presence of added protein the probe was shifted to a higher molecular sized complex consistent with formation of a protein-RNA complex. Addition of an HuR antibody resulted in a supershift (Fig. 3*B*, *lane 3*), confirming the presence of HuR in the complex. No shift was observed when antibodies against either AUF1 or HuB were used in the supershift assay. In Fig. 3*B, lane 4*, a 50-fold excess of unlabeled probe was used to compete for HuR binding. Thus, in an *in vitro* assay HuR forms an mRNP complex with the ARE from the 3′-UTR of C/EBPβ.

Rapid Formation of Nuclear HuR-C/EBPβ Complexes and Their Translocation to the Cytosol—To determine whether the C/EBPβ message was present in an mRNP complex with HuR, immunoprecipitations were performed on both nuclear and cytosolic extracts prepared at 0 and 30 min after the addition of the differentiation inducers. As shown in Fig. 4*A*, Western blot analysis indicates that the HuR protein was detected in the nuclear fractions at both time points. However, the decreased nuclear presence of the HuR protein 30 min after induction of differentiation is readily apparent. Analysis of the mRNA present in the immunoprecipitated mRNP complex shown in Fig. 4*B*, indicates that, prior to the addition of the differentiation inducers, HuR-C/EBPβ complexes could not be detected. However, within 30 min of addition of the inducers C/EBPβ mRNA was found to be present in the immunoprecipitated complex. Examination of the cytosol for the presence of these complexes over the same time period demonstrates the presence of HuR protein in the cytosol (Fig. 4*C*) with an increase detectable at 30 min after the induction of differentiation, consistent with translocation. In Fig. 4*D* the presence of the C/EBPβ mRNA in the immunoprecipitated complex at the 30-min time point is demonstrated. The data presented in Fig. 4 support a model in which treatment of the preadipocytes with the differentiation inducers results in formation of a HuR-C/EBPβ message complex in the nucleus. This is followed by translocation to the cytosol and represents a very early event in the differentiation

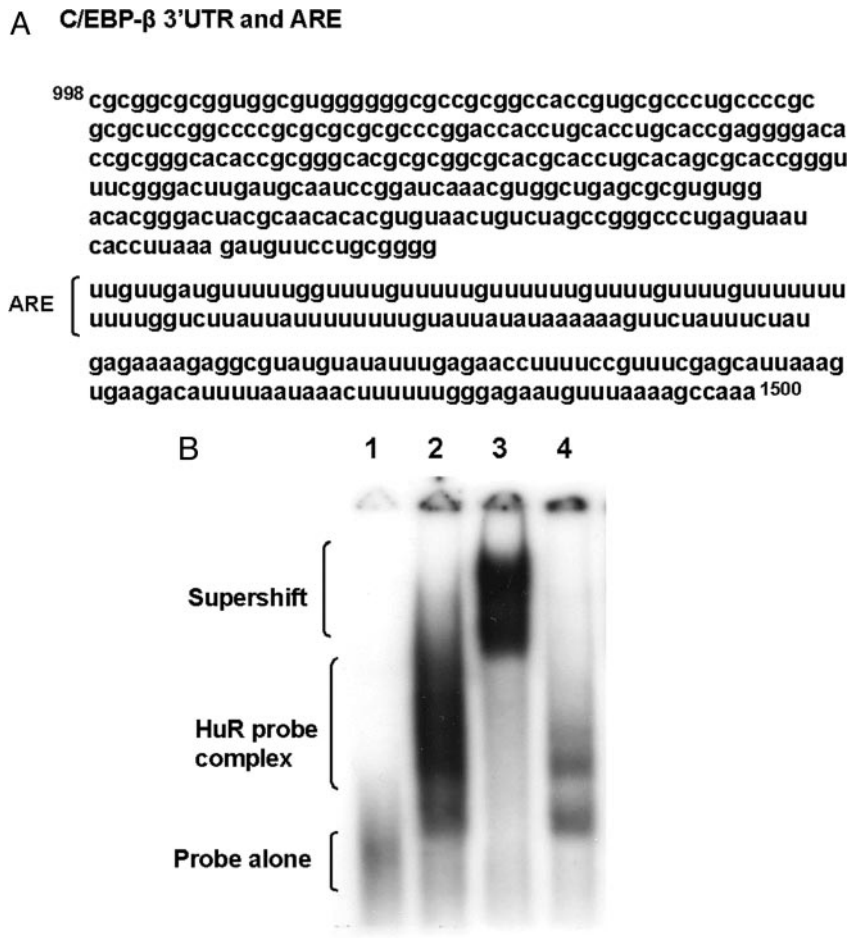

FIG. 3. **The C/EBPβ mRNA is a ligand for HuR *in vitro*.** *Panel A*, the 3′-UTR and ARE of the C/EBPβ mRNA. *Panel B*, cytosolic extracts and a radiolabeled probe corresponding to the C/EBPβ 3′-UTR were used to perform RNA gel shift and supershift analysis. *Lane 1*, probe alone; *lane 2*, probe plus 10 μg adipocyte cytosolic extract; *lane 3*, supershift of complex (formed as in *lane 2*) using 3A2-HuR monoclonal antibody; *lane 4*, binding performed in the presence of a 50-fold excess of unlabeled probe. These data are representative of gel shifts performed in duplicate with three individually prepared sets of cell extracts.

program. Examination of the translocation at later time points (Fig. 5) indicates the presence of C/EBPβ mRNA in the complex at 2 h, with a stronger signal evident at the 48-h time point. By day 8 after induction, a HuR-C/EBPβ complex can no longer be detected, consistent with diminished expression of the transcription factor (data not shown).

To assess the specificity of the selection of the C/EBPβ message by HuR, we examined for the presence of cyclin D1 mRNA in the immunoprecipitates. Cyclin D1 was selected because it has a strong ARE in the 3′-UTR and is expressed during the differentiation program on the same time frame as C/EBPβ (38). In addition, we examined for the presence of GATA-3 mRNA, a message containing no ARE. Neither cyclin D1 nor GATA3 mRNA were detected (data not shown). The data demonstrate a highly regulated HuR selection of the C/EBPβ mRNA as a very early event in the differentiation process.

Effect of HuR Depletion on Adipocyte Differentiation—Using Dharmacon SMARTpool™ four-oligo siRNA technology, we developed an approach to suppress HuR protein content in the 3T3-L1 cells. The study used two controls, our transfection agent, Lipofectamine 2000, alone, and a Dharmacon non-targeting four-oligo pool (Sc). As detailed under "Experimental Procedures," cells at 80% confluency were transfected with 100 nM oligo for 6 h, at which time the media was replaced with Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. Twenty-four hours later, two monolayers were combined to generate one confluent monolayer, which was absolutely essential for induction of differentiation. Eighteen hours later (48 h after the initial transfection), the cells were exposed to the differentiation inducers. Preliminary experiments demonstrated that HuR protein levels at the time of treatment with the differentiation inducers were decreased by 70 ± 12% (n = 4) relative to the controls. HuR content remained decreased for at least 5 days, at which time HuR protein began to increase gradually as the long term effectiveness of the treatment diminished by day 10. The observed phenotype is presented and characterized in Fig. 6. The data presented in Fig. 6, *A* and *B* display the results of monitoring the cells for the appearance of morphological differentiation. At 5 days post-induction of differentiation, both Lipofectamine 2000 and Sc controls were accumulating lipid droplets as demonstrated by phase-contrast microscopy of both unstained (Fig. 6*A*) and Oil Red O-stained cells (Fig. 6*B*). The decreased magnification of the Oil Red O-stained cells represents a larger field and demonstrates the uniformity of the lipid accumulation in the controls. However, the cells treated with HuR siRNAs retained the preadipocyte morphology at this time. In companion day 5 monolayers treated identically to those displayed in Fig. 6, *A* and *B*, Western blot analysis was performed for HuR and C/EBPβ (Fig. 6, *C* and *D*). The data displayed in Fig. 6*C* demonstrate a 65% suppression of HuR protein relative to both Lipofectamine 2000 and Sc controls. Similar results were observed with C/EBPβ (Fig. 6*D*), where the protein content of both the liver-activating protein (LAP) and liver-inhibitory protein (LIP) forms of the transcription factor are decreased markedly relative to controls. Examination of the expression of C/EBPα and C/EBPδ as well as PPARγ (Fig. 6*E*) demonstrates that these transcription factors were also suppressed in the HuR-depleted cells (for comparison, note that the β-tubulin present as a loading control in the Sc lane is decreased relative to the Lipofectamine 2000 lane by 40% in Fig. 6). These results provide a strong correlation between the levels of HuR and C/EBPβ expression

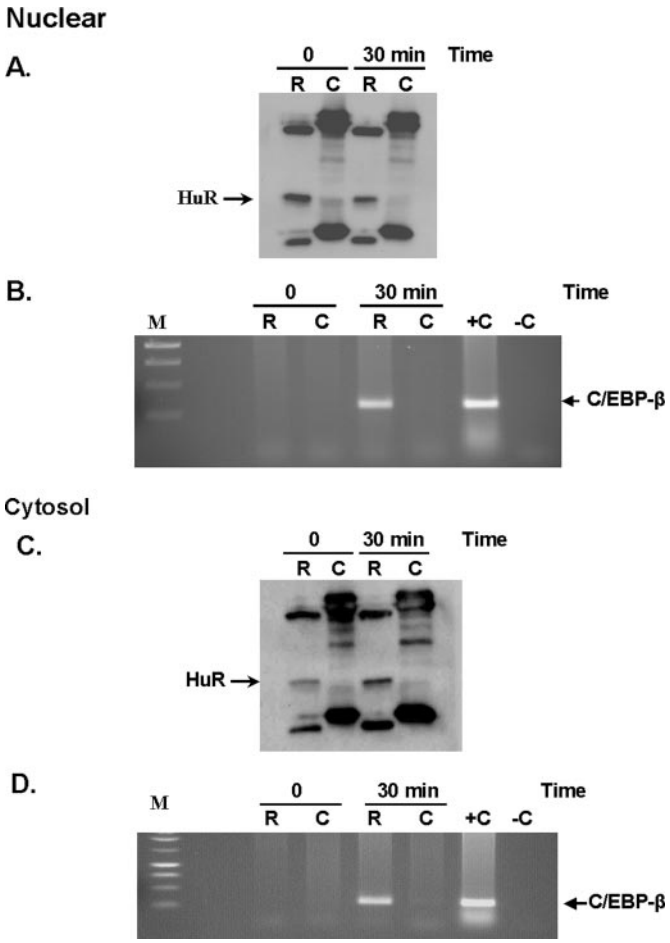

FIG. 4. **Rapid formation of nuclear HuR-C/EBPβ complexes and their translocation to the cytosol.** *Panel A*, time course Western analysis confirms the presence of HuR in the immunoprecipitate. Shown is the preadipocyte at time 0 and 30 min after exposure to the differentiation inducers. *R*, immunoprecipitate performed with the HuR monoclonal antibody; *C*, immunoprecipitate performed with control antibody (*arrow* indicates HuR. The other bands are the heavy and light immunoglobulin chains from the immunoprecipitations. *Panel B*, using six identical immunoprecipitates, RNA was extracted and subjected to reverse transcription PCR analysis for the presence of C/EBPβ (*arrow* indicates C/EBPβ). Designations and times are as stated in *panel A*. *M*, molecular weight marker. These data are representative of four separations performed with similar results. *Panel C* demonstrates the presence of HuR protein in the cytosol with an increase detectable at 30 min after the induction of differentiation, consistent with translocation. *Panel D* shows the presence of the C/EBPβ mRNA in the immunoprecipitated complex at the 30-min time point. The designations and times for *panels C* and *D* are the same as for *panels A* and *B*.

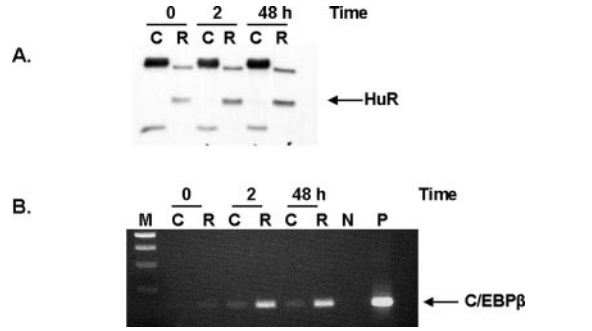

FIG. 5. **C/EBPβ-HuR complex formation at extended times.** Nomenclature and designations are identical to those in Fig. 4 with the following additions: *P*, positive control (total day 4 adipocyte RNA); *N*, negative (water) control, *Panel A*, time course Western analysis at times 0, 2, and 48 h after exposure to the differentiation inducers. *Panel B*, reverse transcription PCR analysis for the presence of C/EBPβ. These data are representative of at least four separations performed with similar results.

displayed in Fig.6, *C* and *D* and the acquisition of the adipocyte morphology displayed in *panels A* and *B* of Fig. 6. Most importantly, these data are consistent with the cellular protein content of HuR controlling C/EBPβ expression and, thus, the acquisition of the adipocyte phenotype.

DISCUSSION

Our results describe the constitutive expression of the RNA-binding protein HuR in the 3T3-L1 preadipocytes with a modest 3-fold increase in protein content with respect to differentiation. At confluence in the preadipocyte, the majority of HuR protein is retained in the nucleus. However, 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. When HuR expression was reduced 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. These observations are consistent with maintenance of HuR protein content and the ability of the cells to express C/EBPβ and progress through the differentiation program. Importantly, work by others has demonstrated that the differentiation process can be blocked with the reagents leptomycin B (42) or 5-amino-4-imidazolecarboxamide (AICAR) (43). However, although in both studies differentiation was blocked, the C/EBPβ protein was expressed at normal (42) to above normal levels (43). This suggests that suppression of HuR expression results in an inhibition of differentiation a step earlier than the accumulation of C/EBPβ protein. Our data 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 that, in turn, are recognized by specific nucleoporins (20, 23, 26, 39). However, the *C/EBPβ* gene has no introns and must utilize an alternative export mechanism, which is likely mediated by HuR recognition of the 3′-ARE (39). 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 myogenesis or neural development (33, 34). Previous work from our laboratory 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 (29, 40). These data suggested that an endogenous member of the Hu family may play a role in the temporal control of the differentiation process. The data presented herein would suggest that HuR serves that purpose.

The choice of a ligand mRNA by HuR is a selective process. Neither cyclin D1, an ARE-containing mRNA expressed with the same kinetics as C/EBPβ (38), nor GATA3, a message without an ARE, was present in the mRNP complexes. Wang *et al.* (32) have reported that HuR present in extracts prepared from serum-stimulated RKO cells did not bind to the cyclin D1 ARE in gel shift assays. This was all the more interesting, as previous studies have demonstrated that recombinant HuR would bind to the cyclin D1 ARE in similar gel shift assays, indicating that the metabolic/hormonal state of the cell may control the selection of ligands by the HuR protein. We would suggest that the tight temporal control of specific mRNA selec-

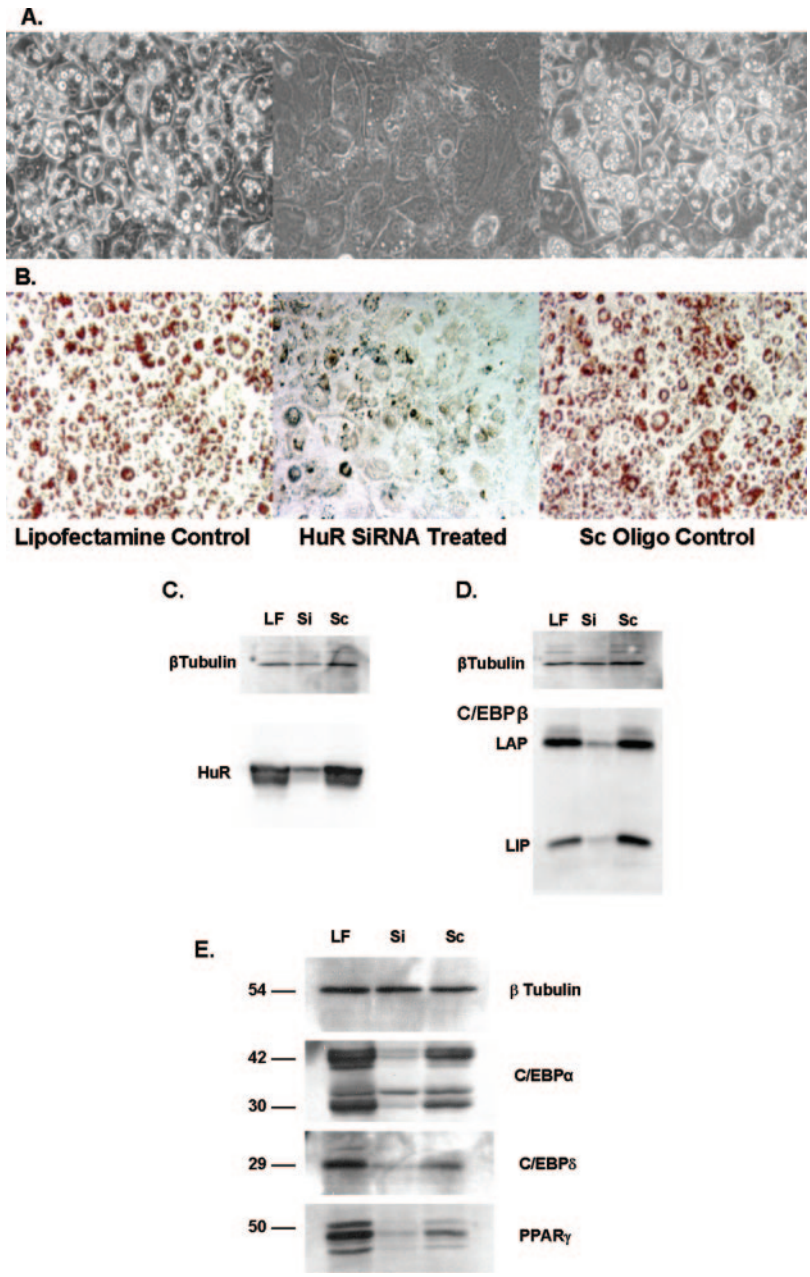

FIG. 6. **Depletion of HuR by siRNA treatment attenuates the differentiation process.** After exposure to the transfection protocol described under "Experimental Procedures," two monolayers in each treatment group were trypsinized and combined to generate immediate confluency. Eighteen hours later (48 h after the initial transfection), the cells were exposed to the differentiation protocol and monitored daily for the accumulation of lipid. *Panels A* and *B*, morphological analysis of the differentiation process. The pictures displayed present the cells at day 5 post-induction of differentiation. *Panel A*, phase-contrast 40× microscopy of Lipofectamine 2000-treated (*left*), HuR siRNA-treated (*middle*), and the Sc RNA-treated cells (*right*). *Panel B*, the wells displayed in *panel A*. after staining with Oil Red O, viewed at a 20× magnification. *Panels C* and *D*, Western blot for HuR and C/EBPβ, respectively, from individual wells corresponding to the three treatments described above. *LF*, Lipofectamine 2000; *Si*, four-oligo siRNA; *LAP*, liver-activating protein; *LIP*, liver-inhibitory protein. The blots were probed with β-tubulin, the protein content of which does not change over the time course of this experiment, as a loading/normalization control. *Panel E*: Western blot (as above) for C/EBPα, C/EBPδ, and PPARγ. It is important to note that the quantification indicates that, relative to the Lipofectamine 2000 (*LF*) *lane*, there is a 40% decrease in the β-tubulin content of the *Sc lane*. The data shown in *panels A* and *B* of this figure are representative of an experiment performed three times with identical results. The results in *panel E* are representative of an experiment performed twice with identical results.

tion 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.

Upon differentiation, a new distribution of HuR protein is established with as much as 40% of the total remaining in the cytosol, long after the expression of C/EBPβ has diminished. Our previous work has established that, in the fully differentiated adipocytes, the GLUT1 glucose transporter mRNA is a ligand for HuR and that other ligand mRNAs are selected with respect to the time course of differentiation (41).[2] We suggest that HuR not only functions in establishing the adipocyte phenotype but aids in its maintenance.

Acknowledgments—We thank Bentley Cheatham and Jack Keene for the discussions that aided in the development of our system.

REFERENCES

1. Patel, Y. M., and Lane, M. D. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 1279–1284
2. Tang, Q. Q., Otto, T. C., and Lane, M. D. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 44–49
3. Zhang, J.-W., Klemm, D. J., Vinson, C., and Lane, M. D. (2004) *J. Biol. Chem.* **279,** 4471–4478
4. Shepherd, P. R., Gnudi, L., Tozzo, E., Yang, H., Leach, F., and Kahn, B. B. (1993) *J. Biol. Chem.* **268,** 22243–22246
5. Gnudi, L., Shepherd, P. R., and Kahn, B. B. (1996) *Proc. Nutr. Soc.* **55,** 191–199
6. Van, R. L., Bayliss, C. E., and Roncari, D. A. (1976) *J. Clin. Investig.* **58,** 699–704
7. Tang, Q. Q., Otto, T. C., and Lane, M. D. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 850–855
8. Tang, Q.-Q., and Lane, M. D. (1999) *Genes Dev.* **13,** 2231–2241
9. Yeh, W.-C., Cao, Z., Classon, M., and McKnight, S. L. (1995) *Genes Dev.* **9,** 168–181
10. Cole, K. A., Harmon, A. W., Harp, J. B., and Patel, Y. M. (2004) *Am. J. Physiol.* **286,** C349–C354
11. Rosen, E. D., Walkey, C. J., Puigserver, P., and Spiegelman, B. M. (2000) *Genes Dev.* **14,** 1293–1307
12. Hwang, C., Loftus, T., Mandrup, S., and Lane, M. (1997) *Annu. Rev. Cell Dev. Biol.* **13,** 231–259
13. Christy, R. J., Kaestner, K. H., Geiman, D. E., and Lane, M. D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 2593–2597
14. Tang, Q. Q., Jiang, M. S., and Lane, M. D. (1999) *Mol. Cell. Biol.* **19,** 4855–4865
15. Zhu, Y., Qi, C., Korenberg, J. R., Chen, X. N., Noya, D., Rao, M. S., and Reddy, J. K. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7921–7925
16. Clarke, S. L., Robinson, C. E., and Gimble, J. M. (1997) *Biochem. Biophys. Res. Commun.* **240,** 99–103

[2] K. Gantt, U. Atasoy, and P. H. Pekala, manuscript in preparation.

17. Tang, Q. Q., Jiang, M. S., and Lane, M. D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 13571–13575
18. Lu, J.-Y, and Schneider, R. J. (2004) *J. Biol. Chem.* **279,** 12974–12979
19. Ma, W. J., Cheng, S., Campbell, C., Wright, A., and Furneaux, H. (1996) *J. Biol. Chem.* **271,** 8144–8151
20. Fan, X. C., and Steitz, J. A. (1998) *EMBO J.* **17,** 3448–3460
21. Myer, V. E., Fan, X. C., and Steitz, J. A. (1997) *EMBO J.* **16,** 2130–2139
22. Levy, N. S., Chung, S., Furneaux, H., and Levy, A. P. (1998) *J. Biol. Chem.* **273,** 6417–6423
23. Akamatsu, W., Fujihara, H., Mitsuhashi, T., Yano, M., Shibata, S., Hayakawa, Y., Okano, H. J., Sakakibara, S.-I., Takanok, H., Takano, T., Takahashi, T., Noda, T., and Okano, H. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 4625–4630
24. Peng, S. S., Chen, C. Y., Xu, N., and Shyu, A. B. (1998) *EMBO J.* **17,** 3461–3470
25. Brennan, C. M., and Steitz, J. A. (2001) *Cell. Mol. Life Sci.* **58,** 266–277
26. Gallouzi, I. E., and Steitz, J. A. (2001) *Science* **294,** 1895–1901
27. Shaw, G., and Kamen, R. (1986) *Cell* **46,** 659–667
28. Jacobson, A., and Peltz, S. W. (1996) *Annu. Rev. Biochem.* **65,** 693–739
29. Jain, R. G., Andrews, L. G., McGowan, K. M., Pekala, P. H., and Keene, J. D. (1997) *Mol. Cell. Biol.* **17,** 954–962
30. Antic, D., Lu, N., and Keene, J. D. (1999) *Genes Dev.* **13,** 449–461
31. Li, H., Park, S., Kilburn, B., Jelinek, M. A., Henschen-Edman, A., Aswad, D. W., Stallcup, M. R., and Laird-Offringa, I. A. (2002) *J. Biol. Chem.* **277,** 44623–44630
32. Wang, W., Caldwell, M. C., Lin, S., Furneaux, H., and Gorospe, M. (2000) *EMBO J.* **19,** 2340–2350
33. van der Giessen, K., Di-Marco, D., Clair, E., and Gallouzi, I. E. (2003) *J. Biol. Chem.* **278,** 47119–47128
34. Tenenbaum, S. A., Lager, P. J., Carson, C. C., and Keene, J. D. (2002) *Methods* **26,** 191–198
35. Deleted in proof
36. Stephens, J. M., and Pekala, P. H. (1992) *J. Biol. Chem.* **267,** 13580–13584
37. Bernlohr, D. A., Bolanowski, M. A., Kelly, T. J., Jr., and Lane, M. D. (1985) *J. Biol. Chem.,* **260,** 5563–5567
38. Morrison, R., and Farmer, S. R. (1999) *J. Biol. Chem.* **274,** 17088–17097
39. Brennan, C. M., Gallouzi, I. E., and Steitz, J. A. (2000) *J. Cell Biol.* **151,** 1–13
40. Gantt, K. R., Jain, R. G., Dudek, R. W., and Pekala, P. H. (2004) *Biochem. Biophys. Res. Commun.* **313,** 619–622
41. Gantt, K., Atasoy, U., Harrison, R., Cheatham, B., and Pekala, P. H. (2003) in *Proceedings of the Keystone Conference on RNA Trafficking, Transport, and Processing, Snowbird, UT, April 5–10, 2003* (Ephrussi, A., Mattaj, I. W., and Dreyfuss, G.), p. 47, Keystone Symposia, Silverthorne, CO
42. Sprott, K. M., Chumley, M. J., Hanson, J. M., and Dobrowsky, R. T. (2002) *Biochem. J.* **365,** 181–191
43. Habinowski, S. A., and Witters, L. (2001) *Biochem. Biophys. Res. Commun.* **286,** 852–856

An Early Event in Adipogenesis, the Nuclear Selection of the CCAAT Enhancer-binding Protein β (C/EBPβ) mRNA by HuR and its Translocation to the Cytosol

Kira Gantt, Joy Cherry, Raleigh Tenney, Vesna Karschner and Phillip H. Pekala

J. Biol. Chem. 2005, 280:24768-24774.
doi: 10.1074/jbc.M502011200 originally published online April 29, 2005
