MOLECULAR AND CELLULAR BIOLOGY, July 2003, p. 4991–5004 Vol. 23, No. 14
0270-7306/03/$08.00+0 DOI: 10.1128/MCB.23.14.4991–5004.2003

Role of HuR in Skeletal Myogenesis through Coordinate Regulation of Muscle Differentiation Genes

Angélica Figueroa,[1] Ana Cuadrado,[1] Jinshui Fan,[2] Ulus Atasoy,[3] George E. Muscat,[4] Pura Muñoz-Canoves,[5] Myriam Gorospe,[2]* and Alberto Muñoz[1]

Instituto de Investigaciones Biomédicas 'Alberto Sols,' Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, 28029 Madrid,[1] and Centro de Regulación Genómica, 08003 Barcelona,[5] Spain; Laboratory of Cellular and Molecular Biology, NIA-IRP, National Institutes of Health, Baltimore, Maryland 21224,[2] and Duke University School of Medicine, Durham, North Carolina 27710[3]; and Institute for Molecular Bioscience, University of Queensland, St Lucia, QLD 4072, Australia[4]

Received 26 March 2003/Returned for modification 23 April 2003/Accepted 1 May 2003

In this report, we investigate the role of the RNA-binding protein HuR during skeletal myogenesis. At the onset of myogenesis in differentiating C2C12 myocytes and in vivo in regenerating mouse muscle, HuR cytoplasmic abundance increased dramatically, returning to a predominantly nuclear presence upon completion of myogenesis. mRNAs encoding key regulators of myogenesis-specific transcription (myogenin and MyoD) and cell cycle withdrawal (p21), bearing AU-rich regions, were found to be targets of HuR in a differentiation-dependent manner. Accordingly, mRNA half-lives were highest during differentiation, declining when differentiation was completed. Importantly, HuR-overexpressing C2C12 cells displayed increased target mRNA expression and half-life and underwent precocious differentiation. Our findings underscore a critical function for HuR during skeletal myogenesis linked to HuR's coordinate regulation of muscle differentiation genes.

Skeletal muscle cells have proven to be an excellent model system for defining the molecular mechanisms involved in the decision between continued proliferative growth and tissue differentiation (27). During muscle differentiation, proliferating myoblasts permanently withdraw from the cell cycle and fuse to become postmitotic, multinucleated myotubes with a contractile phenotype that will ultimately mature into myofibers (29). These morphogenic changes are accompanied by specific alterations in the patterns of muscle-specific genes expressed (27). Particularly important among them are two groups of transcription factors: the MyoD family, comprising MyoD, Myf5, myogenin, and myogenic regulatory transcription factor 4 (MRF-4), and the myocyte enhancer factor-2 family (28). In turn, these proteins regulate the transcription of muscle-specific genes required to establish myoblast identity and control their terminal differentiation. MyoD and Myf5 are expressed in proliferating myoblasts, while increased abundance of myogenin and p21 (cyclin-dependent kinase inhibitor) marks a stage in which myoblasts are destined for fusion and terminal differentiation into myotubes (12, 35, 36). Regenerating adult muscle shares many features of embryonic muscle differentiation. Adult muscle fibers express undetectable levels of MRFs, except for MRF4, but MRF expression is induced during injury-induced skeletal muscle regeneration. The in vivo expression of MyoD and myogenin during regeneration is similar to that observed in developing limbs (16). Skeletal muscle regeneration after injury is characterized by the proliferation and differentiation of muscle precursor cells, followed by their fusion to form new or restored myofibers. A tight regulation of differentiation and regeneration is therefore critical for the production of functional muscle.

Transcriptional as well as posttranscriptional mechanisms critically contribute to regulating gene expression patterns during cellular processes such as proliferation, differentiation, the stress response, immune cell activation, growth arrest, and cell death. Among posttranscriptional events, mRNA turnover is emerging as a critical paradigm of gene regulation (13, 31, 32). Even small differences in mRNA half-life can rapidly alter its abundance and, consequently, the amount of protein expressed. The mechanisms determining mRNA turnover, generally believed to involve RNA-binding proteins that recognize specific RNA sequences, have become the focus of intense investigation in recent years. Best characterized among the RNA sequences influencing mRNA stability are AU-rich elements (AREs), usually found in the 3′ untranslated region (UTR) of labile mRNAs (11, 42), such as those encoding cytokines (interferon and interleukins), cell cycle regulatory genes (p21, cyclin A, cyclin B1, and cdc25 genes), growth factors (granulocyte-macrophage colony-stimulating factor and vascular endothelial growth factor), apoptosis-related genes (*bcl*-2), and proto-oncogenes (c-*fos* and c-*myc*). While many RNA-binding proteins have been described that selectively recognize and bind to AREs, their influence on mRNA turnover is best known for the embryonic lethal abnormal vision (ELAV) and AUF1 proteins (7, 24, 43).

Initially described in *Drosophila melanogaster*, ELAV protein was found to be essential for proper differentiation of the nervous system (9). Genetic, molecular, and biochemical studies have suggested that ELAV's mammalian counterparts may likewise participate in regulating tissue growth and differentiation. HuR is expressed ubiquitously, while HuC and HuD are specifically expressed in neuronal tissues and HuB is expressed

* Corresponding author. Mailing address: Box 12, LCMB, NIA-IRP, NIH, 5600 Nathan Shock Dr., Baltimore, MD 21224. Phone: (410) 558-8443. Fax: (410) 558-8386. E-mail: myriam-gorospe@nih.gov.

in neuronal tissues and in testes and ovaries (2, 7). Several recent reports support a role for ELAV proteins in neuronal differentiation in mammalian systems. In rat PC12 cells, overexpression of HuB, HuC, or HuD induced neuronal differentiation features such as neurite outgrowth in the absence of nerve growth factor, while diminished HuD expression reduced neurite outgrowth in the presence of NGF (1, 21). ELAV proteins possess three RNA recognition motifs that mediate binding with high affinity and specificity to ARE-containing target mRNAs (7). ELAV proteins have been reported to target mRNAs encoding proteins important for differentiation (i.e., neurofilament N and GAP-43 [2, 3, 5, 33]) and for growth and proliferation (i.e., p21, cyclin A, cyclin B1, c-*fos*, and VEGF) by increasing their stability, translation, or both (14, 19, 20, 25, 37).

The precise mechanisms whereby HuR mediates mRNA stabilization are still poorly understood. HuR is predominantly localized in the nucleus of most unstimulated cells but can translocate to the cytoplasm upon cell stimulation, a process that has been linked to the stabilization of many target mRNAs (14, 15, 22, 30, 37). The association of HuR with PP32, APRIL, and SETα/β proteins is believed to influence HuR export from the nucleus and possibly also to modulate HuR's affinity for its target mRNAs (8). Although PP32 and SETα/β are known to inhibit protein phosphatase 2A, AMP-activated kinase is the only kinase thus far directly implicated in the regulation of HuR cytoplasmic levels and the consequent stabilization of HuR target mRNAs (40).

In the present investigation, we study the role of HuR in myogenesis using in vitro and in vivo models. HuR was almost exclusively nuclear in actively proliferating, undifferentiated C2C12 myoblasts, but it became strikingly abundant in the cytoplasm upon induction of differentiation by insulin, transferrin, and selenium (ITS), remained elevated in the cytoplasm for the duration of the differentiation process, and returned to a predominantly nuclear presence upon the completion of cell differentiation. Similarly, in an animal model of skeletal muscle regeneration, which largely resembles embryonic muscle differentiation, cytoplasmic HuR increased in regenerating myofibers, declining upon completion of the process. These events coincided temporally with the expression of myogenin and MyoD. During C2C12 cell differentiation, HuR was found to target mRNAs encoding myogenin, p21, and MyoD, all of them being pivotal regulators of muscle cell differentiation; such increased association was linked to increased stability of the corresponding mRNAs. Importantly, overexpression of HuR in C2C12 cells increased the expression and half-life of target mRNAs and accelerated C2C12 differentiation. We propose that HuR plays a critical role in myogenesis in vitro and in vivo.

MATERIALS AND METHODS

Cell culture and transfection. C2C12 murine myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum and antibiotics (Gibco-BRL, Gaithersburg, Md.). To induce differentiation, cells (80 to 90% confluence) were cultured in DMEM containing ITS for up to 5 days (120 h). Transient transfection of C2C12 cells with pEGFP-HuR (37) was carried out using Lipofectamine (Invitrogen, Carlsbad, Calif.) for 5 h. After removal of the transfection medium, cells were incubated in either 10% fetal bovine serum or serum-free DMEM containing ITS for the times indicated.

Nuclear run-on, Northern blot, and Western blot analyses. Northern blot analysis from C2C12 cells was carried out by using standard procedures. Probes were prepared with [α-^{32}P]dATP by either random-primed labeling of PCR-amplified fragments corresponding to the coding regions of myogenin, MyoD, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or by end labeling of oligonucleotides recognizing the 18S rRNA (used to monitor the loading and transfer of Northern blot samples) and mouse p21 mRNA (17). For mRNA half-life assessments, actinomycin D (ActD) (2.5 μg/ml) was added, and total RNA was prepared at the times indicated. mRNA half-lives were calculated after measurement of Northern blot signals, normalization to 18S signals, plotting on logarithmic scales (line graphs), and calculation of the time required for a given transcript to be reduced to half of its initial signal intensity (bar graphs). Nuclear run-on assays were carried out as previously described (13, 17), using 1 μg of purified PCR product per slot.

For Western blotting, cytoplasmic and nuclear fractions were prepared as described previously (37). Twenty micrograms of whole-cell protein, 10 μg of cytoplasmic lysate, and 10 μg of nuclear lysate were routinely utilized for Western blot analysis. After transfer, membranes were hybridized with antibodies recognizing either HuR (monoclonal anti-HuR antibody, Molecular Probes, Eugene, Oreg.), myogenin (monoclonal antibody from Developmental Studies Hybridoma Bank, Baltimore, Md.), β-tubulin (monoclonal antibody from Oncogene Research Products, San Diego, Calif.), histone deacetylase 8 and MyoD (Santa Cruz), or myosin heavy chain (Developmental Studies Hybridoma Bank, Iowa City, Iowa). Following incubation with the appropriate secondary antibodies, signals were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech).

In vitro transcription. DNA templates for in vitro transcription of various regions of the myogenin, p21, and MyoD mRNAs were synthesized by PCR using reverse transcribed cDNA, except for myogenin, which was prepared using plasmid pEMSV-myogenin as template. All 5′ primers contained the T7 RNA polymerase promoter (T_7, CCAAGCTTCTAATACGACTCACTATAGGGA GA). For amplification of the myogenin coding region (GenBank no. D90156), encompassing nucleotides 387 to 719, primers used were T_7-GCTGCCTAAAG TGGAGATC and GGAAGGCAACAGACATATC; for amplification of the myogenin 3′ UTR (encompassing positions 769 to 1471), the primers used were T_7-GCCATCACTTCTGTAGCAG and GATATCAACCAGTCTTTATTC. For amplification of the MyoD coding region (GenBank no. NM010866), encompassing positions 261 to 831, the primers used were T_7-GAGACAGCAGA CGACTTCTATG and CGCTGTAATCCATCATGCCATC; for amplification of the MyoD 3′ UTR, encompassing positions 1241 to 1762, the primers used were T_7-GACACTCTTCCCAACTGTCC and GCACTACACAGCATGCCT. p21 fragments were amplified as reported previously (37). All PCR templates were purified from agarose gels. RNA transcripts were synthesized using T7 RNA polymerase in the presence of either [α-^{32}P]UTP or biotinylated CTP and purified as described previously (37, 38).

Binding assays: RNase T1 selection, agarose gel shift assay, UV cross-linking, RNA electrophoretic mobility shift assay (REMSA), and supershift REMSA. Glutathione *S*-transferase (GST) and GST-HuR were prepared as described previously (20). Reaction mixtures (20 μl) contained 50 mM Tris-HCl [pH 7.0], 0.25 mg of tRNA/ml, 0.25 mg of bovine serum albumin/ml, 10 to 20 fmol of labeled RNA (100,000 to 600,000 cpm), and purified GST or GST-HuR. After 10 min of incubation at 37°C, 5 U of RNase T1 was added to each reaction for an additional 10 min. Reaction mixtures were filtered through nitrocellulose, and bound HuR-RNA complexes were extracted with phenol-chloroform. RNA was then precipitated, dissolved in formamide, denatured (65°C, 2 min), and size separated by 10% polyacrylamide–50% urea gel electrophoresis. Gels were dried and exposed to film. Agarose gel shift assays were done as described previously (20).

For UV cross-linking assays, REMSAs, and supershift REMSAs, binding reactions and electrophoresis to visualize complexes were carried out as previously described (37). For supershift assays, 0.5 μg of either anti-HuR or anti-p38 antibodies (Santa Cruz) was used.

Immunofluorescence. Cells were fixed in cold methanol. Endogenous HuR was visualized in samples incubated with anti-HuR antibody (Molecular Probes) and then with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG; 115-095-003; Jackson Laboratories). Enhanced green fluorescent protein (EGFP) and chimeric EGFP-HuR (37) were visualized directly. Confocal microscopy was performed with a Bio-Rad Laboratories MRC-1024 laser scanning microscope equipped with an Axiovert 100 microscope (ZEISS) at an excitation wavelength of 488 nm.

Biotin pull-down. Binding of proteins (40 μg of cytoplasmic lysate) to biotinylated transcripts (2 μg) was performed in the presence of RNase inhibitor (5 Prime→3, Prime, Inc., Boulder, Colo.) for 30 min at room temperature. Com-

plexes were isolated with paramagnetic streptavidin Dynabeads (Dynal, Oslo, Norway) for 30 min, washed with phosphate-buffered saline, and subjected to Western blot analysis to detect HuR.

Analysis of muscle regeneration in mice. Prior to injury, mice (C57BL/6, 8 to 12 weeks old) were anesthetized by an intraperitoneal injection of dormicum and droperidol. Regeneration of skeletal muscle was induced by intramuscular injection of 400 μl of 10 μM cardiotoxin (Latoxan, France), as described previously (10), in the right gastrocnemius muscle, using contralateral intact muscles as control. Morphological and biochemical examinations were performed at 0, 0.4, 3, 9, and 20 days after injury, using three animals per time point. For Western blotting, gastrocnemius muscles were dissected, sectioned, weighed, and pounded in an ice-cold Potter tube containing 0.1 mM Tris-HCl [pH 7.6], 2 mM EDTA, and 0.4% Triton X-100; extracts were centrifuged for 20 min at 4°C at 12,000 × *g*, and supernatants were used for Western blotting. For histological analysis, after cervical dislocation of the mice, muscles were removed, dissected, frozen in liquid nitrogen, and stored at −80°C prior to sectioning. Transverse cryostat sections (10 μm thick) were stained with hematoxylin-eosin (HE) to visualize cellular structures. Endogenous HuR was visualized by immunofluorescence as described above.

IP of endogenous HuR-mRNA complexes. The association of endogenous HuR with endogenous mRNAs encoding myogenin, MyoD, and p21 was assessed in C2C12 cells undergoing differentiation using a method previously described (34). Twenty million C2C12 cells were collected per time point, and lysates were used for immunoprecipitation (IP) for 4 h at room temperature in the presence of excess (30 μg) immunoprecipitating antibody (either a mouse monoclonal anti-HuR antibody, 3A2 [a generous gift of J. A. Steitz], or an IgG1 isotype control antibody [B&D Life Sciences]). RNA in IP material was used in reverse transcription-PCR (RT-PCR) to detect the presence of mRNAs encoding myogenin, MyoD, and p21 using Superscript One-Step RT-PCR system (Invitrogen). Primer pairs for PCR amplification of the coding regions of myogenin and MyoD mRNAs are described above; amplification of the mouse p21 mRNA (GenBank no. U24173) encompassing positions 26 to 737 was performed using primers TGTCAGAGTCTAGGGGAATTGG and AGGTTTGGAGAC TGGGAGAG. PCR amplification was carried out as follows: 1 min at 94°C, 1 min at 55°C, and 1 min at 68°C, for 20 cycles (myogenin) or 24 cycles (MyoD, p21). PCR products were visualized on 1.5% agarose gels by ethidium bromide staining.

RESULTS

C2C12 muscle cell differentiation is associated with increased stabilization of mRNAs encoding myogenin, MyoD, and p21. The mouse myoblast cell line C2C12 has been used extensively to study muscle cell differentiation (27). As shown in Fig. 1A, treatment with ITS causes actively proliferating, undifferentiated myoblasts to differentiate into distinct myotubes, a process lasting approximately 5 days (120 h). In keeping with previous reports (29), myogenin expression changed dramatically throughout the differentiation period (Fig. 1B). In order to investigate the extent to which its expression was controlled through altered transcription, we carried out nuclear run-on analysis (Fig. 1C); myogening gene transcription was elevated at the late times examined. Given the relatively modest transcriptional increase of myogenin expression (~eightfold) and the robust ITS-triggered induction in myogenin mRNA (>70-fold), we hypothesized that mRNA stabilization might act in concert with increased transcription to achieve the strong induction in myogenin expression (Fig. 1).

Examination of the myogenin mRNA revealed a relatively long 3′ UTR containing AREs (depicted as shaded regions in Fig. 3A, below). Given that AREs are linked to changes in mRNA stability, we set out to investigate whether myogenin expression was regulated through altered mRNA turnover during ITS-triggered C2C12 cell differentiation. First, we carried out measurements of myogenin mRNA half-life using ActD-based assays. Briefly, at various times after ITS addition (0, 4, 8, 12, 24, 48, and 120 h), ActD was added to block new mRNA transcription, and RNA was prepared at successive times thereafter (0, 1, 2, 4 h, etc). Northern blot analysis served to monitor the rate of clearance of the myogenin transcript (Fig. 1D) and thus calculate the myogenin mRNA half-life at various times following addition of ITS. The myogenin mRNA half-life varied throughout the differentiation period: it increased during the early stages of differentiation, having an apparent half-life of 2.5 h at the first time point examined (4 h after addition of ITS), a half-life of 5 h at 8 h of ITS treatment, and a half-life of 10.5 h at 12 h of ITS treatment. Myogenin mRNA stability appeared to be maximal at around 24 h into ITS-induced differentiation, when its apparent half-life was greater than 20 h. At subsequent time points, the myogenin mRNA stability declined, exhibiting a half-life of 7 h at 48 h into the differentiation process and a half-life of 4 h by 120 h (5 days) of ITS treatment, when differentiation was complete and myotubes were formed.

Several other genes that are known to participate in myocyte differentiation also bear AREs in their 3′ UTRs. Two such genes, namely, the p21 and MyoD genes, were found to encode mRNAs that displayed similar alterations in stability throughout the differentiation process. As shown in Fig. 2A, p21 and MyoD mRNA levels increased during the early phases of C2C12 differentiation. MyoD mRNA half-life appeared to be maximal (~4.5 h) at 24 h into differentiation, while p21 mRNA half-life seemed to reach its greatest value (~12 h) at 48 h of differentiation; in each case, mRNA stability declined thereafter (Fig. 2B). In summary, the stability of mRNAs encoding key differentiation-associated genes increased during the early stages of differentiation of C2C12 myocytes.

The 3′ UTRs of myogenin, MyoD, and p21 are HuR targets during C2C12 muscle cell differentiation. The mechanisms underlying the stabilization of mRNAs encoding these three differentiation-associated genes were further investigated by examining whether these mRNAs were targets of RNA-binding proteins that could alter their stability in a differentiation-regulated manner. To this end, different regions of each cDNA were amplified by PCR using 5′ primers that contained the T7 RNA polymerase promoter sequence. PCR-generated templates were used for in vitro synthesis of transcripts corresponding to various regions of myogenin, MyoD, and p21 mRNAs (Fig. 3A; see Materials and Methods). Radiolabeled transcripts corresponding to either the coding region or the 3′ UTR of myogenin, MyoD, and p21 were incubated with cytoplasmic lysates from C2C12 cells undergoing differentiation. As shown in Fig. 3B, the myogenin 3′ UTR transcript, but not a transcript encompassing its coding region, formed complexes with proteins present in cytoplasmic lysates of C2C12 cells; such complexes were readily detectable by REMSA. Compared with cytoplasmic proteins from untreated cells, cytoplasmic proteins from cells undergoing differentiation produced markedly more abundant binding to myogenin 3′ UTR. Increased association with cytoplasmic proteins was also seen when using the MyoD 3′ UTR and p21 3′ UTR probes. Of note, the MyoD 3′ UTR transcript was poorly digested by RNase T1, presumably due to the extensive secondary structure of this RNA. Despite our best efforts, much radiolabeled transcript remained undigested after incubation with RNase T1, even in control samples without lysate (Fig. 3B, lane f),

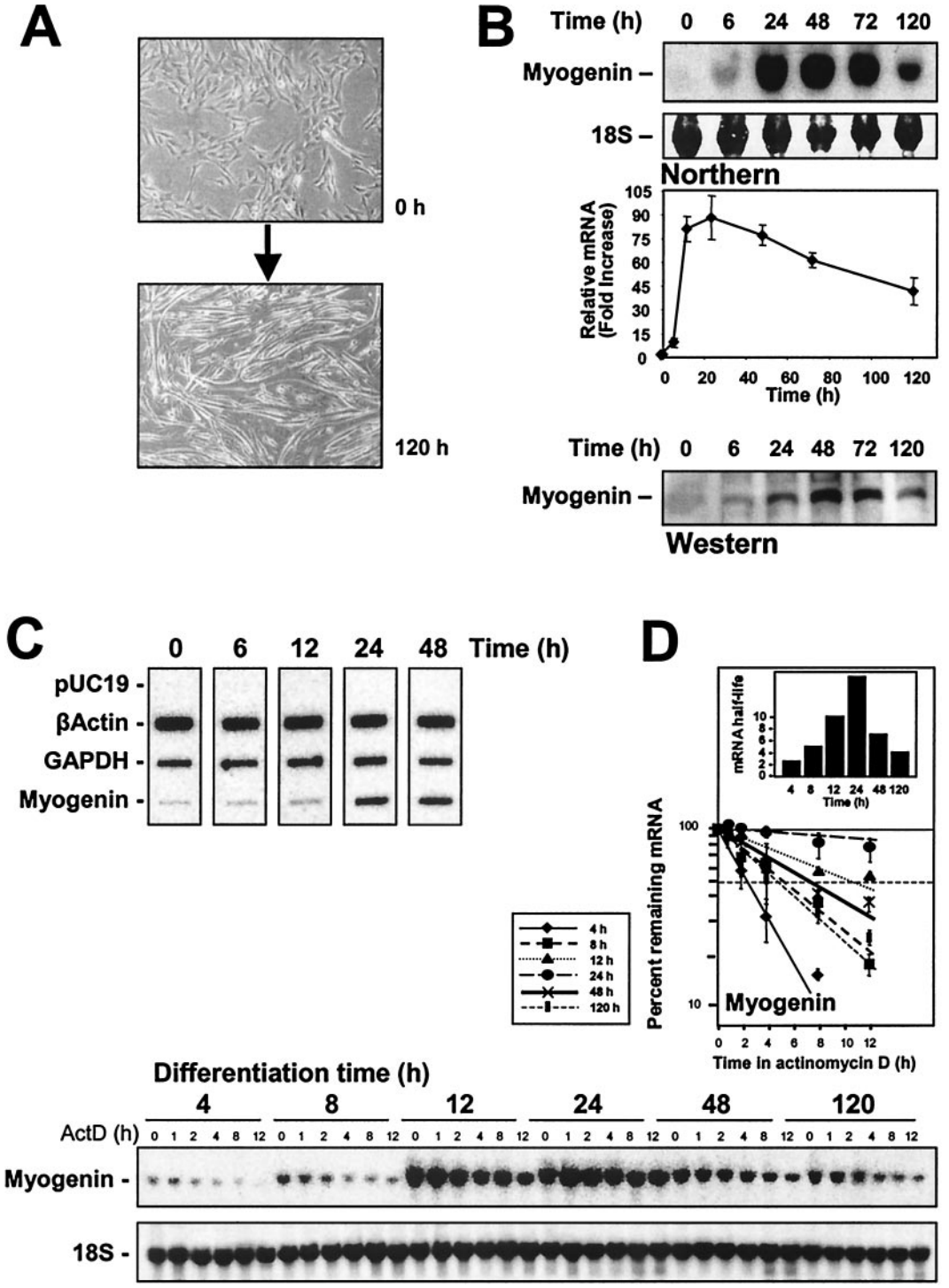


FIG. 1. Myogenin expression and mRNA stability are altered during differentiation of C2C12 muscle cells. (A) Asynchronously growing C2C12 cells (top) were subjected to differentiation using ITS. After 120 h (5 days) in continuous presence of ITS, cultures were fully differentiated and displayed abundant myotubes (bottom). (B) Top, Northern blot analysis of myogenin expression in C2C12 cells undergoing differentiation. 18S rRNA signals served to control for RNA loading. The graph depicts myogenin mRNA signal intensities (relative to 18S signals) shown as the means ± standard errors of the means from three independent Northern blot analyses. Bottom, Western blot analysis of myogenin expression in C2C12 cells undergoing differentiation for the times indicated. All lanes on the Western blot were loaded equally. (C) Nuclear run-on analysis to monitor transcription in C2C12 cells undergoing differentiation for the time periods shown. Nascent, radiolabeled RNA was hybridized to 1 μg of PCR-generated and slot blotted cDNAs; pUC19 plasmid was included as control for nonspecific hybridization. (D) Assessment of myogenin mRNA stability during C2C12 cell differentiation. mRNA half-lives were calculated after treatment of cells with 2.5 μg of ActD/ml and preparation of RNA at the times indicated; myogenin mRNA signals were measured, normalized to 18S rRNA signals, and plotted on a logarithmic scale. Horizontal dashed lines, 50% of untreated. Data represent the means ± standard deviations from three experiments. Bar graphs depict mRNA half-life values during C2C12 differentiation.

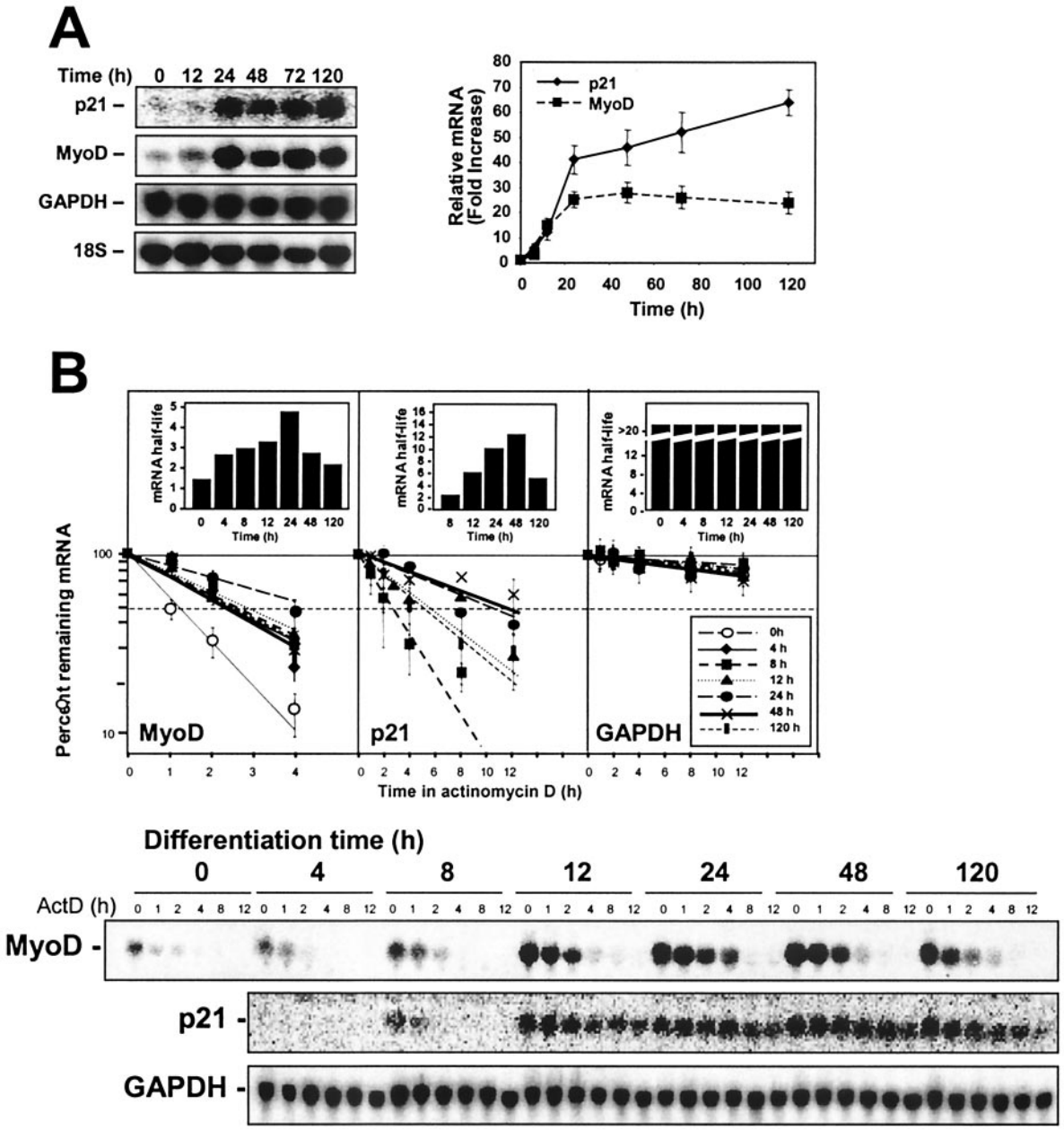
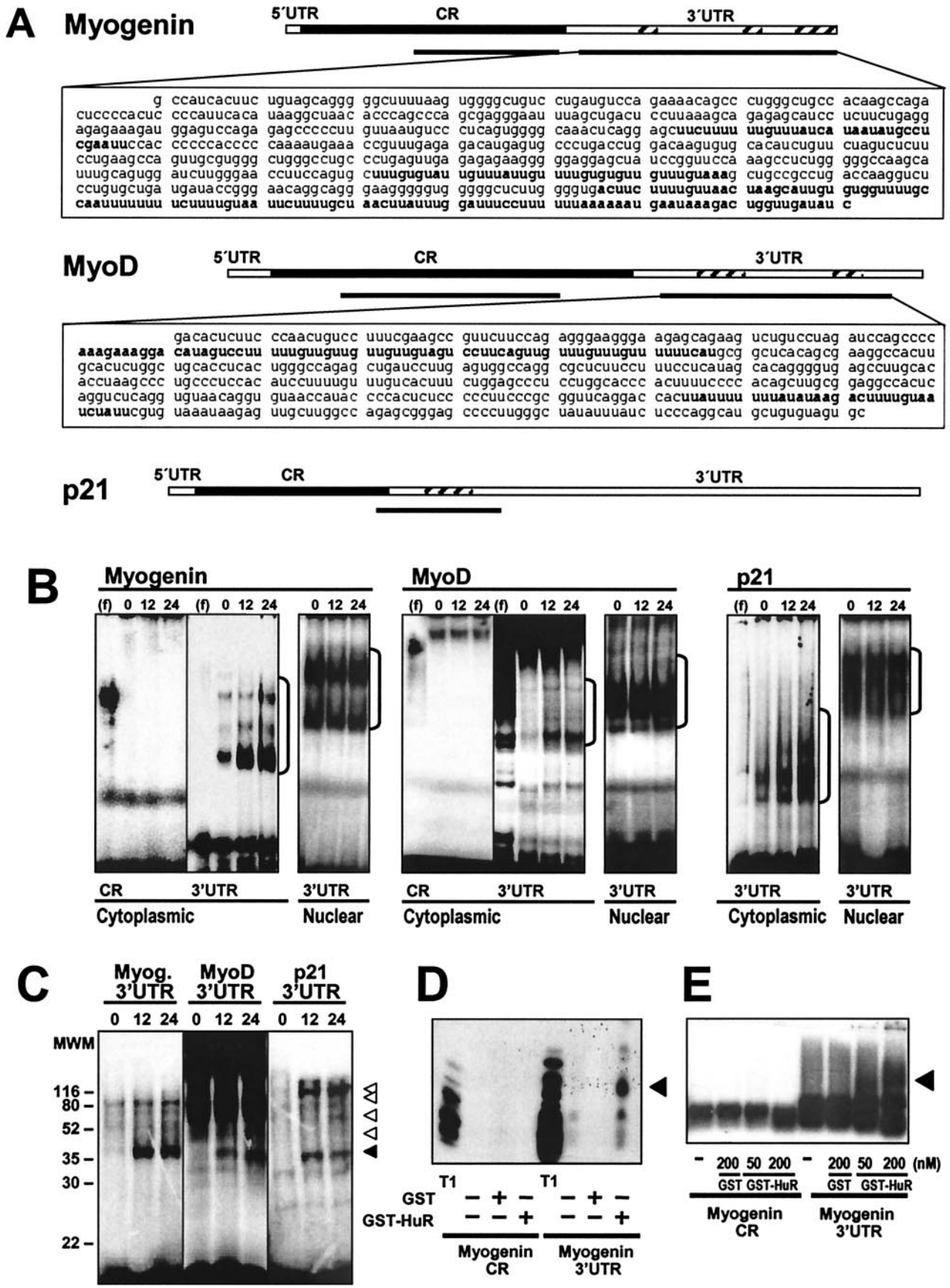


FIG. 2. Half-lives of mRNAs encoding the MyoD and p21 muscle differentiation-associated genes change during C2C12 myocyte differentiation. (A) Northern blot analysis of MyoD, p21, and GAPDH expression in C2C12 cells undergoing differentiation for the times shown. The graph depicts the intensities of mRNAs encoding p21 and MyoD (relative to 18S), shown as the means ± standard errors of the means from three independent Northern blot analyses. (B) The stability of mRNAs encoding MyoD, p21, and GAPDH was calculated as explained in the legend for Fig. 1D.

hampering our ability to perform certain types of assays using this transcript (see below). None of the transcripts encompassing coding regions formed discernible complexes with proteins present in cytoplasmic lysates (Fig. 3B and previously reported results for p21 [37]). As shown earlier for other treatment conditions and transcripts (37, 39), REMSA using nuclear lysates and radiolabeled 3′ UTR transcripts revealed extensive binding in each case, but binding was unaffected by C2C12 cell differentiation (Fig. 3B). In conclusion, the 3′ UTRs of these three mRNAs were bound by cytoplasmic proteins from C2C12 cells in a differentiation-dependent manner.

Next, we sought to identify the cytoplasmic protein(s) forming complexes with the 3′ UTR transcripts of myogenin, MyoD, and p21. RNA-protein complexes forming with radiolabeled transcripts were cross-linked and visualized after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Several complexes were readily visible, with some forming irrespective of the cell differentiation stage and others exhibiting


A
Myogenin
5′UTR
CR
3′UTR
g ccaucacuuc uguagcaggg ggcuuuuaag uggggcuguc cugaugucca gaaaacagcc cugggcugcc acaagccaga
cuccccacuc cccauucaca uaaggcuaac acccagccca gcgagggaau uuagcugacu ccuuaaagca gagagcaucc ucuucugagg
agagaaagau ggaguccaga gagccccuu guuaaugucc cucaguggg caaacucagg agcuucuuuu uuguuuauca uaauaugccu
cgaauuccac cccccacccc caaaaugaaa ccguuugaga gacaugagug cccugaccug gacaagugug cacaucuguu cuagucucuu
ccugaagcca guugcguggg cugggccugc ccugaguuga gagagaaggg ggaggagcua uccgguucca aagccucugg gggccaagca
uuugcagugg aucuugggaa ccuuccagug cuuuguguau uguuuauugu uuugugugu guuuguaaag cugccgccug accaaggucu
ccugugcuga ugauaccggg aacaggcagg gaaggggugg ggggcucuug gggugacuuc uuuuguuaac uaagcauugu gugguuuugc
caauuuuuuu ucuuuuguaa uucuuuugcu aacuuauuug gauuuccuuu uuuaaaaaau gaauaaagac ugguugauau c
MyoD
5′UTR
CR
3′UTR
gacacucuuc ccaacugucc uuucgaagcc guucuuccag agggaaggga agagcagaag ucuguccuag auccagcccc
aaagaaagga cauaguccuu uuuguuguug uuguuguagu ccuucaguug uuuguuuguu uuuucaugcg gcucacagcg aaggccacuu
gcacucuggc ugcaccucac ugggccagag cugauccuug aguggccagg cgcucuuccu uuccucauag cacaggggug agccuugcac
accuaagccc ugcccuccac aucccuuuugu uugucacuuu cuggagcccu ccuggcaccc acuuuucccc acagcuugcg gaggccacuc
aggucucagg uguaacaggu guaaccauac cccacucucc cccuucccgc gguucaggac cacuuauuuu uuuauauaag acuuuuguaa
ucuauucgug uaaauaagag uugcuuggcc agagcgggag ccccuugggc uauauuuauc ucccaggcau gcuguguagu gc
p21
5′UTR
CR
3′UTR
B
Myogenin
MyoD
p21
(f) 0 12 24 (f) 0 12 24 0 12 24
CR 3′UTR 3′UTR
Cytoplasmic Nuclear
C
Myog. 3′UTR
MyoD 3′UTR
p21 3′UTR
0 12 24 0 12 24 0 12 24
MWM
116 –
80 –
52 –
35 –
30 –
22 –
D
T1 T1
GST – + – – + –
GST-HuR – – + – – +
Myogenin CR
Myogenin 3′UTR
E
– 200 50 200 – 200 50 200 (nM)
GST GST-HuR GST GST-HuR
Myogenin CR
Myogenin 3′UTR

a differentiation-induced pattern. Among the latter group was a single common band appearing with all three transcripts, with a size of approximately 36 to 38 kDa (Fig. 3C). We hypothesized that this band corresponded to the RNA-binding protein HuR based on the following facts: (i) HuR is 36 kDa, (ii) HuR has high affinity for AREs akin to those existing in the 3′ UTRs of myogenin and MyoD, (iii) HuR and other ELAV proteins have been reported to play an important role in the differentiation of neuronal tissues, and (iv) HuR binds the p21 mRNA (37). To test this possibility directly, recombinant GST or GST-HuR was incubated with radiolabeled myogenin transcripts and then subjected to RNase T1 selection assays (see Materials and Methods). After filtering through nitrocellulose, protected fragments were seen only after incubation of the myogenin 3′ UTR's transcript with GST-HuR, but not after incubations containing either GST or a transcript corresponding to the myogenin coding region (Fig. 3D). Radiolabeled myogenin 3′ UTR and GST-HuR formed complexes that could also be visualized in agarose gel retardation assays (Fig. 3E).

In order to assess whether endogenous HuR bound these target mRNAs, we performed supershift REMSA analysis with cytoplasmic lysates from differentiating C2C12 cells and radiolabeled transcripts encompassing the 3′ UTRs of myogenin and p21. REMSA complexes increased during the early phases of differentiation and declined at the completion of differentiation (120 h), returning to the levels seen in untreated cells (Fig. 4A). The presence of HuR in REMSA complexes was ascertained by supershift analysis using an anti-HuR antibody. As shown in Fig. 4A, such analysis revealed that HuR was indeed present in the radiolabeled complexes, and the abundance of supershifted complexes increased in a differentiation-dependent manner (although it is important to note that supershift REMSA analyses are not strictly quantitative). The specificity of the supershifted bands was confirmed through use of a control antibody recognizing the mitogen-activated protein kinase p38, which failed to supershift radiolabeled complexes. Given that the MyoD 3′ UTR transcript was poorly digested by RNase T1 (Fig. 3B), we were unable to perform supershift REMSA for this mRNA. Therefore, in order to ascertain whether the MyoD 3′ UTR was a target of HuR, we used two alternative approaches, as described below.

First, the association of HuR with these target mRNAs was assessed using biotinylated transcripts encompassing the regions indicated (Fig. 4B). After incubation with cytoplasmic lysates of C2C12 cells undergoing differentiation, biotinylated RNAs encompassing the 3′ UTRs of myogenin, MyoD, and p21 effectively pulled down HuR, as detected by Western blot analysis (see Materials and Methods). As shown, association of HuR with biotinylated transcripts was very low in untreated cultures, increased with cell differentiation, and decreased upon completion of differentiation. Control biotinylated transcripts, such as an RNA encompassing the myogenin coding region (and other coding regions, not shown), did not pull down HuR (Fig. 4B). Second, we examined the binding of endogenous HuR to endogenous mRNAs encoding myogenin, MyoD, and p21 in C2C12 cells. IP of HuR and associated endogenous mRNAs was tested using either a monoclonal anti-HuR antibody (3A2) or an isotype-matched IgG1 (Fig. 4C), and the IP material was assessed for presence of myogenin, MyoD, and p21 mRNAs by RT-PCR (see Materials and Methods). As shown in Fig. 4D, the three transcripts were remarkably abundant in material obtained from IP using anti-HuR antibody but were either undetectable or much less abundant (at 0 h for MyoD and 6 h for MyoD and p21) in IP material obtained with a control, isotype-matched IgG1. Moreover, binding of HuR to its target mRNAs increased as C2C12 cell differentiation progressed.

It is important to note the existence of lag time between the detection of HuR binding to target transcripts, which occurs very rapidly (Fig. 3 and 4), and the increase in the specific target transcript's half-life, which reaches its maximum by 12 to 24 h (Fig. 1 and 2). For example, it is possible that HuR binding to a target RNA does not by itself cause immediate mRNA stabilization and may require transport to a particular subcellular compartment or association with another cellular factor(s).

Together, these findings demonstrate that HuR associates with mRNAs encoding myogenin, MyoD, and p21 in vitro and in vivo in a differentiation-dependent manner and that this association increases during the early stages of differentiation and decreases upon completion of the differentiation process.

The cytoplasmic abundance of HuR increases dramatically during C2C12 cell differentiation. The evidence gathered thus far supports the association of transcripts encoding myogenin, MyoD, and p21 with HuR in the cytoplasms of differentiating C2C12 cells. Given extensive evidence linking HuR's cytoplasmic localization with its mRNA-stabilizing function (7, 22, 37), it was of particular interest to investigate the subcellular localization of HuR throughout the differentiation process. Western blot analysis using whole-cell lysates revealed a moderate

FIG. 3. In vitro binding of HuR to the myogenin 3′ UTR. mRNAs encoding myogenin, MyoD, and p21 form complexes with C2C12 cytoplasmic proteins in a time-dependent fashion. (A) Schematic representation of the mRNAs encoding myogenin, MyoD, and p21, and various transcripts corresponding to the coding region and 3′ UTR used in this study. Boxes, RNA sequences of the 3′ UTR transcripts used; the p21 3′ UTR probe was previously reported (20). (B) REMSA detection of RNA-protein complexes. PCR-amplified cDNA fragments were used as templates to synthesize ^{32}P-radiolabeled RNA probes. Cytoplasmic and nuclear fractions (10 μg each) prepared at the times indicated during C2C12 differentiation were assayed for binding to the indicated RNA probes. Complexes were resolved on 7% native polyacrylamide gels that were subsequently dried and visualized with a PhosphorImager. Brackets indicate inducible RNA-protein complexes. f (free probe), radiolabeled RNA incubated only with RNase T1. (C) Cytoplasmic fractions prepared from differentiating C2C12 cells were incubated with myogenin, MyoD, and p21 3′ UTR transcripts, and the resulting radiolabeled complexes were digested with RNase T1 and subjected to UV cross-linking and electrophoresis through sodium dodecyl sulfate-containing 15% polyacrylamide gels. Upon drying of gels, signals were visualized using a PhosphorImager. Numbers denote sizes (in kDa) of molecular weight markers (MWM). Open arrowheads, radiolabeled complexes; black arrowheads, predicted position of HuR. (D) An RNase T1 selection assay was carried out using myogenin transcripts and 10 nM either GST or GST-HuR. T1, digestions with RNase T1 alone. Arrowhead, protected RNA fragments. (E) Agarose gel retardation assays using myogenin 3′ UTR and the indicated concentrations of either GST or GST-HuR. Arrowhead, protected RNA fragments.

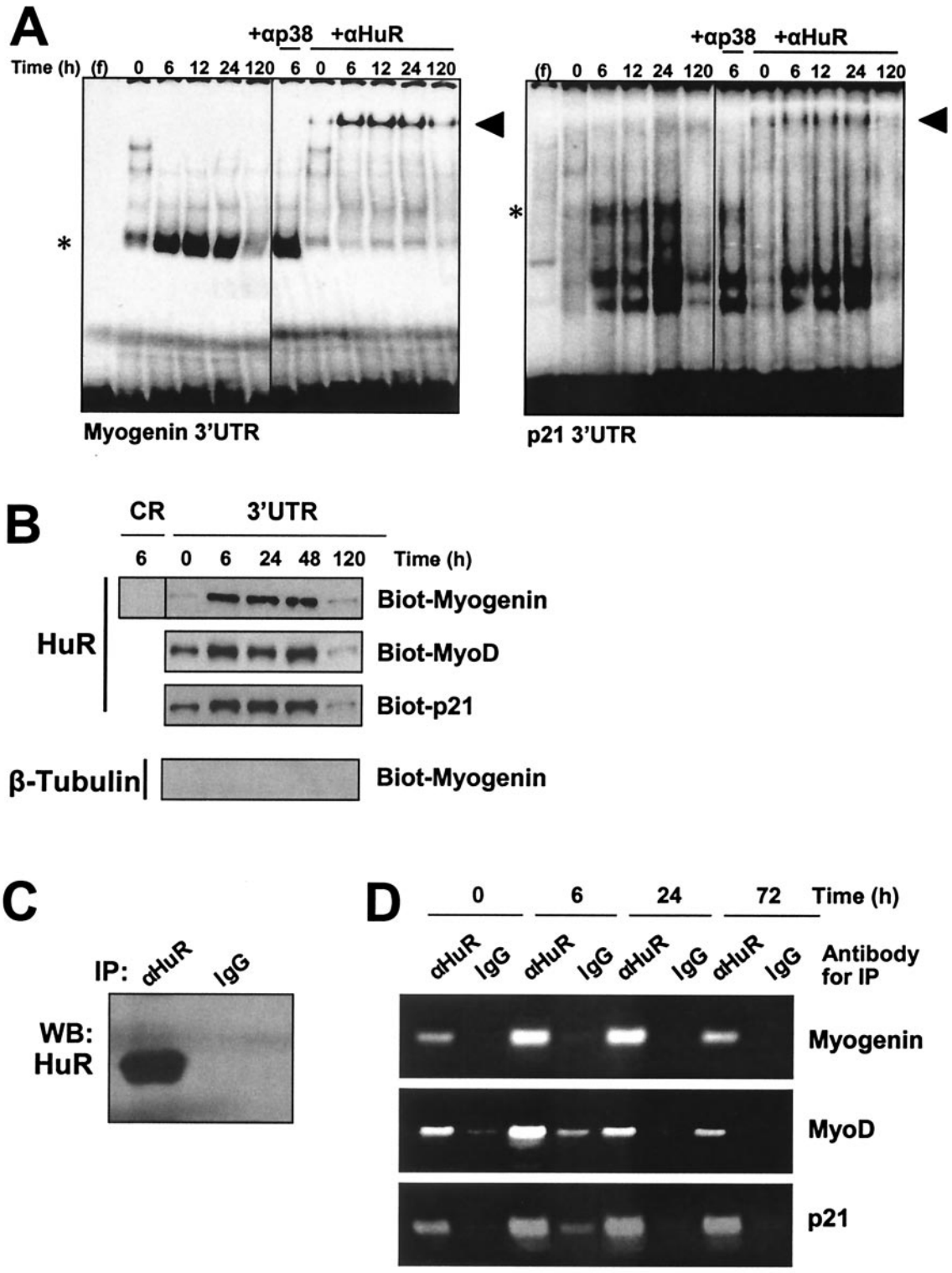


FIG. 4. Endogenous HuR forms differentiation-dependent complexes with recombinant and endogenous transcripts encoding myogenin, MyoD, and p21. (A) Complexes forming with cytoplasmic lysates from C2C12 cells undergoing differentiation for the times indicated and the radiolabeled transcripts shown were assayed for their ability to be supershifted by 0.5 μg of either anti-HuR antibody (+αHuR) or a control antibody recognizing the mitogen-activated protein kinase p38 (+αp38). Arrowheads, HuR-containing supershifted complexes. (B) Biotinylated transcripts corresponding to the 3′ UTRs of each mRNA, as well as the myogenin coding region (CR) (negative control), were incubated with cytoplasmic C2C12 lysates prepared at the times shown after induction of differentiation. Complexes were pulled down using streptavidin-conjugated magnetic beads, and HuR abundance was analyzed by Western blotting. (C) HuR in C2C12 lysates after IP using either anti-HuR antibodies (3A2) or isotype-matched IgG1 was assessed by Western blot analysis (WB) of the IP material. (D) The presence of HuR target mRNAs encoding myogenin, MyoD, and p21 was assessed by RT-PCR analysis of IP material obtained using either anti-HuR or control IgG1 antibodies.

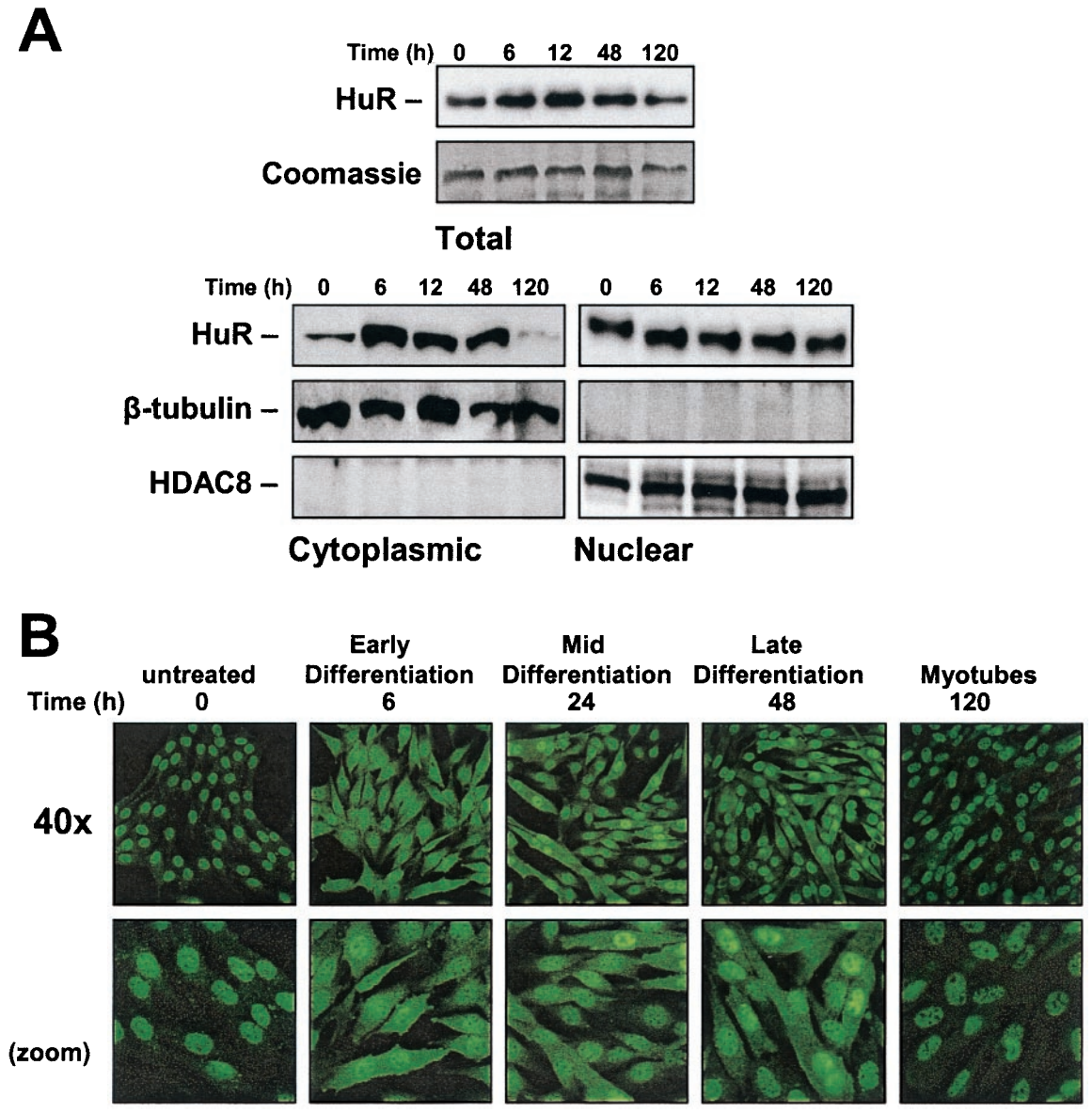


FIG. 5. Effect of C2C12 cell differentiation on the subcellular localization of HuR. (A) Western blot analysis of HuR levels in cytoplasmic (10 μg), nuclear (10 μg), and whole-cell (20 μg) lysates prepared from C2C12 cells that were undergoing differentiation for the times indicated. (B) Immunofluorescence detection of HuR in C2C12 cells throughout the differentiation process. Magnification, ×40; zoom, close-up images.

increase in HuR expression during the early stages of differentiation (by 24 h in ITS); it declined slightly by day 5 (120 h) (Fig. 5A). However, when subcellular fractions were tested, HuR abundance increased remarkably in C2C12 cytoplasms at the onset of differentiation, remained elevated during differentiation, and decreased to almost undetectable levels upon its completion (Fig. 5A). The nuclear abundance of HuR did not fluctuate noticeably throughout differentiation (Fig. 5A). These alterations in HuR's subcellular localization were not due to overall increased expression of HuR, but likely resulted from elevated cytoplasmic transport of HuR, as previously reported (4, 14, 15, 22, 30, 37). Despite the striking changes in cytoplasmic HuR, a correspondingly marked reduction in nuclear HuR was not seen. This is explained by the relative abundance of HuR in each cellular compartment: in untreated C2C12 cells, HuR is about 10-fold more abundant in the nucleus, so even substantial elevations in cytoplasmic HuR produce little change in the nuclear HuR pool. Western blotting of the same membranes to detect nucleus- and cytoplasm-specific proteins (histone deacetylase 8 and β-tubulin, respectively) verified that nuclear proteins did not leak into the cytoplasmic fractions during cell treatment or fractionation and further monitored the even loading and transfer of samples (Fig. 5A). Analysis of histone deacetylase and β-tubulin was routinely performed on all Western blots containing subcellular fractions. Immunohistochemical analysis of HuR expression corroborated these observations. HuR was almost exclusively nuclear in untreated C2C12 cultures, but ITS treatment caused HuR cytoplasmic abundance to increase dramatically at early time points (6 h) and to remain elevated through 24 and

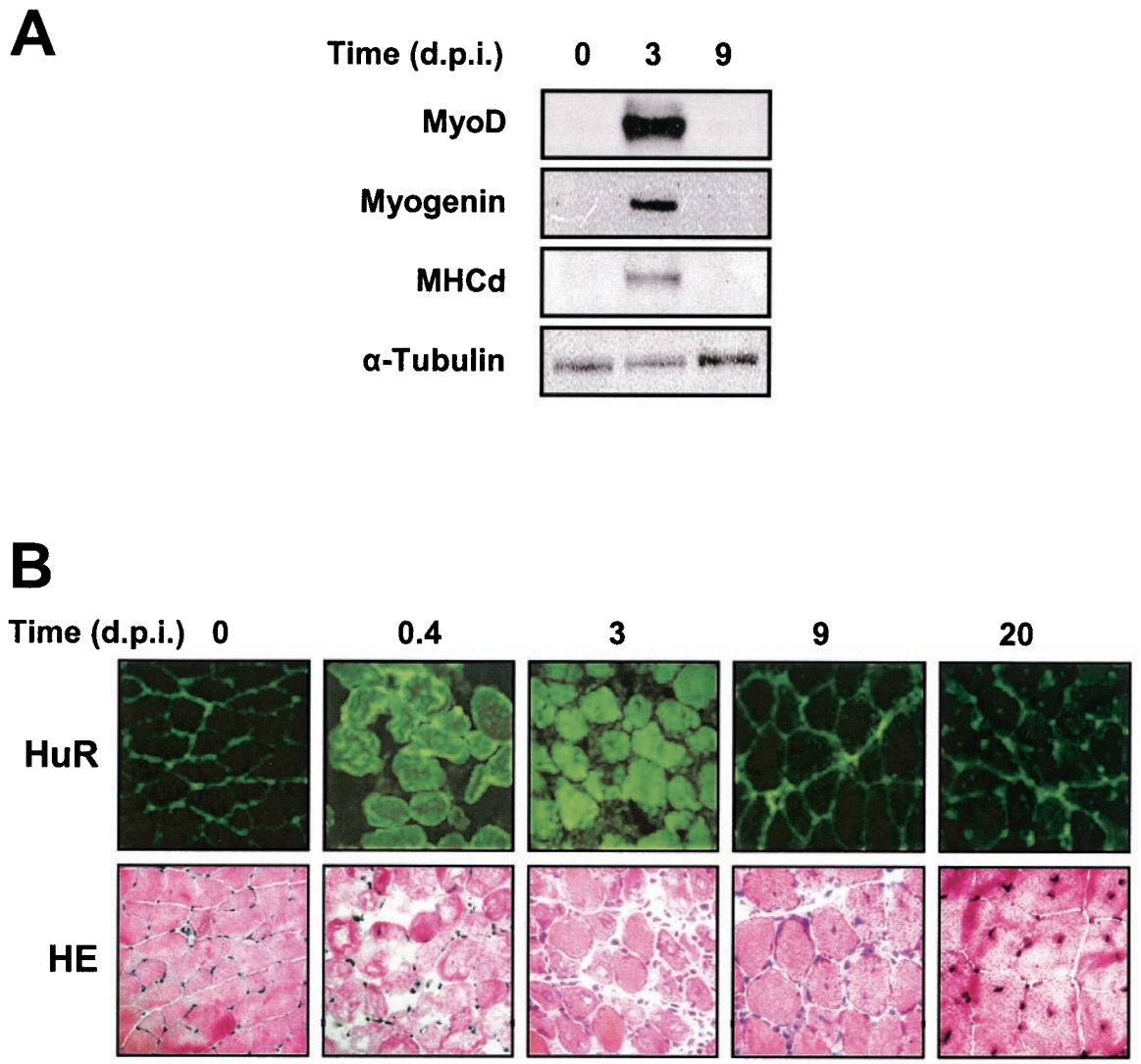


FIG. 6. Cytoplasmic localization of HuR after induced injury in mouse muscle. (A) Muscle lysates from either intact (0 days postinjury [d.p.i.]) or cardiotoxin-injured muscles (3 and 9 d.p.i.) were prepared for Western blot analysis to assess the expression of regeneration markers MyoD, myogenin, and MHCd, as well as loading control α-tubulin. (B) Immunofluorescent detection of HuR in frozen muscle sections prepared at the indicated d.p.i. Sections were stained with HE to monitor adequate progression of the degeneration-regeneration process. Magnification, ×60.

48 h of ITS treatment. HuR returned to a predominantly nuclear localization when differentiation ended (120 h) (Fig. 5B).

Cytoplasmic HuR increases markedly during skeletal muscle regeneration in vivo. The results described thus far suggest a link between HuR and myogenesis using an in vitro model. In order to ascertain whether HuR might be involved in skeletal myogenesis in vivo, we investigated HuR expression and subcellular localization using a mouse model of muscle regeneration. Muscle damage was induced by intramuscular injection of cardiotoxin, and the expression of HuR was investigated in the regenerating muscle at different times postinjury. Injury-induced muscle regeneration was monitored morphologically and by Western blot analysis of muscle samples prepared before injury and 3 and 9 days after injury. As shown, MyoD, myogenin, and the developmental isoform of myosin heavy chain (MHCd, whose expression is restricted to developing muscle in embryos and neonates and to regenerating muscle in adults and which serves as a regeneration marker here) were undetectable in uninjured muscle, increased dramatically 3 days after injury, and returned to basal levels after 9 days, indicating that regeneration was proceeding normally (Fig. 6A). In order to investigate the expression and subcellular localization of HuR during the regeneration process, cryosections of regenerating muscle at different times postinjury were analyzed by immunofluorescence using an anti-HuR antibody (Fig. 6B). HuR was detectable in the nuclei of uninjured myofibers (along the periphery) but not in the cytoplasm of uninjured myofibers. However, HuR expression in the cytoplasm of myofibers increased markedly as early as 6 h after cardiotoxin injection. The most dramatic increase in the cytoplasmic abundance of HuR was seen by 3 days after injury, a time when muscle is actively involved in regeneration. Evidence for ongoing regeneration was apparent in HE-stained preparations,

which exhibited fibrotic infiltrates and inflammatory cells within the enlarged space between myofibers as well as new, small myofibers (Fig. 6B). The heightened cytoplasmic localization of HuR detected on day 3 postinjury coincided with the robust expression of MyoD, myogenin, and MHCd (Fig. 6A). By 9 and 20 days postinjury, as muscle regeneration approached completion, HuR was no longer detectable in the cytoplasm of injured myofibers, and expression of MyoD, myogenin, and MHCd was concomitantly reduced (Fig. 6). The changes in subcellular localization of HuR seen during muscle regeneration parallel those observed in differentiating C2C12 cells and further support a physiological role for HuR during myogenesis.

HuR directly contributes to the implementation of C2C12 differentiation. Strong correlations have thus emerged between HuR cytoplasmic presence during myogenesis, HuR's association with target mRNAs encoding myocyte differentiation genes, and the altered stability of target mRNAs during the C2C12 differentiation. Therefore, we set out to directly assess whether HuR was directly implicated in regulating the expression of differentiation-associated genes and hence the differentiation of C2C12 myoblasts. We overexpressed HuR through transient transfection (Fig. 7A, EGFP-HuR) and examined the expression of target mRNAs by Northern blotting. As shown, steady-state levels of mRNAs encoding myogenin, MyoD, and p21 were higher in cells expressing elevated levels of HuR (EGFP-HuR) than in control, pEGFP-transfected populations (Fig. 7B). This finding is in keeping with numerous examples in which overexpression of HuR resulted in heightened stability of target mRNAs resulting in their increased expression (reviewed in reference 7). Indeed, the half-lives of mRNAs encoding myogenin, MyoD, and p21 increased in cells overexpressing HuR (Fig. 7C).

More importantly, cells overexpressing HuR were capable of forming myotubes earlier in the differentiation process. In EGFP-HuR-expressing C2C12 cells, proportionately more fusion protein was found in the cytoplasm of untreated cultures, but further regulation of its subcellular localization was similar to that of endogenous HuR (not shown). Consequently, in this population, EGFP-positive early myotubes (two- to four-cell fusions) were readily observed by 16 h after transfection, before addition of ITS, and further increased after addition of ITS (Fig. 7D and E). By contrast, no myotubes were seen in control populations transfected with an EGFP-expressing plasmid, although these cells differentiated at the normal pace, with EGFP-positive myotubes beginning to appear 8 h after triggering differentiation by ITS (Fig. 7D and E). Taken together, our studies reveal that HuR is a critical regulator of the expression of key muscle differentiation-associated genes. We propose that it is through its influence on such genes that HuR has a direct impact on myogenesis.

DISCUSSION

In this study, we provide evidence that expression of myogenin, MyoD, and p21 during muscle cell differentiation is subject to regulation through altered mRNA turnover, and we implicate the RNA-binding protein HuR in this process. At the onset of differentiation, the striking increase in steady-state levels of ARE-bearing mRNAs encoding myogenin, MyoD, and p21 was shown to be due to increased transcript stability. Through a series of binding assays using recombinant as well as endogenous proteins, HuR was identified as a major protein binding to the 3′ UTRs of these mRNAs in vitro and to the corresponding endogenous mRNAs in vivo (Fig. 3 and 4). Moreover, the differentiation-dependent associations of HuR with its target mRNAs were found to occur in the cytoplasm of C2C12 cells, the subcellular compartment where HuR is believed to exert its mRNA-stabilizing influence (for review, see reference 7). HuR abundance in C2C12 cytoplasms increased prominently upon induction of myocyte differentiation, remained elevated during the early stages of differentiation, and decreased towards the end of the differentiation period, returning to an almost exclusive nuclear presence when myotubes were formed. Similarly, in an in vivo myogenesis model, injury-induced skeletal muscle regeneration in the mouse also caused a dramatic upregulation of cytoplasmic HuR in regenerating myofibers, coinciding with the de novo expression of myogenic proteins myogenin and MyoD. In support for the hypothesis that HuR regulates the stability of target myogenic mRNAs during muscle cell differentiation were the findings that each mRNA was stabilized with kinetics that coincided with HuR's cytoplasmic presence and that overexpression of HuR enhanced their steady-state levels and half-lives. Importantly, HuR overexpression accelerated the onset of C2C12 differentiation. We postulate that HuR can hasten muscle differentiation through its ability to upregulate the expression of critical differentiation regulatory genes.

As with skeletal muscle development during embryogenesis, a tight control of proliferation and differentiation during skeletal muscle regeneration is required for the production of functional muscle tissue after injury. For example, MyoD is required for satellite cell function in adult skeletal muscle; accordingly, MyoD-deficient mice develop a severe deficiency in their ability to regenerate skeletal muscle (26). Based on our in vitro data supporting HuR's role on the expression of MyoD and myogenin in differentiating C2C12 cells, it is plausible that in vivo, HuR also regulates the stability of myogenic regulators MyoD and myogenin, which are essential for skeletal muscle regeneration.

Our findings support a role for HuR in coordinately regulating the expression of several genes that play pivotal roles in the establishment or maintenance of differentiated muscle tissue. Previous studies by our group and others have demonstrated HuR's role in coordinating the expression of collections of genes involved in the same cellular process. For example, HuR jointly influenced the expression of cyclin A and cyclin B1 during the cell division cycle by coordinately stabilizing their respective mRNAs during the proliferative phases (38). Similarly, maintenance of a "young" cell phenotype in models of in vitro senescence was found to rely on HuR-mediated stabilization of several mRNAs encoding proliferative proteins such as cyclin A, cyclin B1, and c-*fos* (39). The cellular response to stresses such as exposure to UV light likewise causes coordinate changes in the stability of several stress-response genes, like the p21 and gadd153 genes (our unpublished observations), as well as other gadd genes (the gadd34, gadd45, gadd33, and gadd7 genes [18]) and several immediate-early genes, such as c-*fos*, c-*jun*, and c-*myc* (6). Given HuR's increased function by UV (37) and its ability to bind to mRNAs

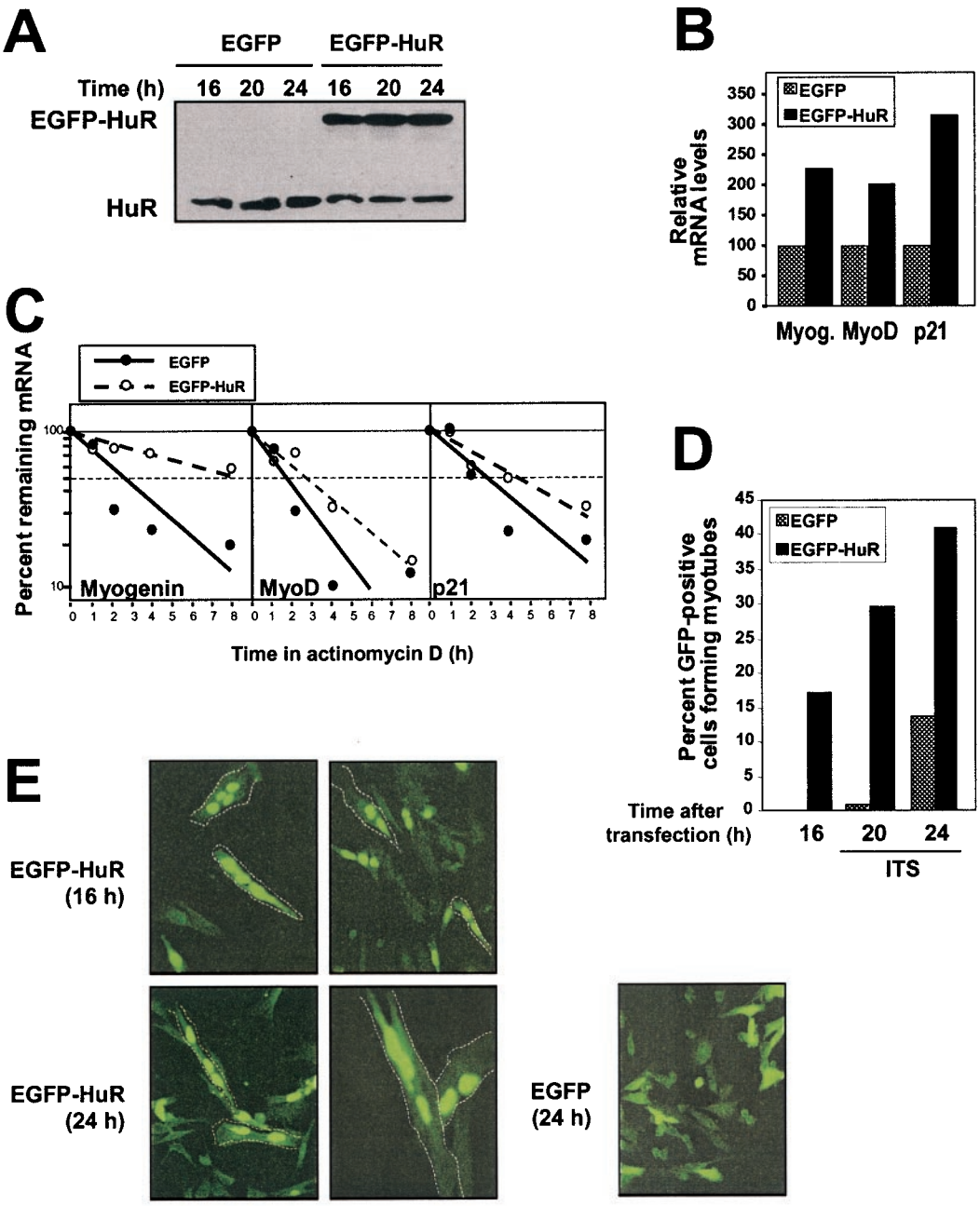


FIG. 7. Overexpression of HuR increases the expression of differentiation-associated genes and accelerates myotube formation. C2C12 cells were transiently transfected with plasmids expressing either EGFP or EGFP-HuR. (A) Western blot analysis of HuR expression using whole-cell lysates. (B) Quantitation of Northern blot signals to measure steady-state mRNA levels 16 h after transfection of either pEGFP or pEGFP-HuR (10 h in the presence of ITS). (C) mRNA half-lives assessed 16 h after transfection (10 h in the presence of ITS), as explained in the legend for Fig. 1D; data are the means of two similar experiments. (D) Following C2C12 transfection, the percentage of EGFP-positive cells forming syncytia (two or more fused myocytes) was assessed in each transfection group. (E) Representative fields depicting early myotube formation in EGFP-HuR-overexpressing C2C12 cells that were transiently transfected as described above. Cultures were examined by phase contrast (data not shown) and fluorescence microscopy; syncytia are indicated with a white discontinuous line. Magnification, ×40.

encoding synchronously regulated genes, HuR may effectively serve as a common endogenous regulator of stress-response gene expression at a posttranscriptional level. In this capacity, the role of HuR within the cellular UV response is akin to that of transcription factors such as activating protein-1, for example, which coordinately increases the transcription of stress-response genes (41).

A more systematic analysis to identify targets of the HuR-related protein HuB during neuronal differentiation of P19 cells was undertaken by Tenenbaum et al. (33). IP of HuB along with HuB-bound target mRNAs, followed by cDNA array hybridization, revealed the existence of subsets of mRNAs whose expression was coordinately regulated at a posttranscriptional level. These investigators have proposed that such

en masse analysis of posttranscriptional mRNA regulation will reveal functional connections between groups of genes that are expressed jointly. They have termed this approach to functional genomics "ribonomics" (34). The hypothesis underlying their model is that mRNAs are organized into groups sharing common functions and structural features. A common RNA-binding protein could thus effectively regulate such functionally and structurally linked mRNA subpopulations during specific cellular processes such as proliferation, differentiation, and the stress response. Keene, Tenenbaum, and coworkers (23, 34) recently proposed that eukaryotic mRNA-protein associations play a role equivalent to that of operons. Prokaryotic operons contain clustered genes that are expressed in single polycistronic mRNAs; their coordinate expression thus allows for efficient synthesis of functionally linked gene products necessary for the organism's response to changes in environmental conditions. Such physical proximity of functionally related genes is not normally seen in the genomes of higher eukaryotes. The proposed posttranscriptional operon model is based on the presence of RNA regulatory elements, typically present in the UTRs of eukaryotic mRNAs, and single RNA-binding proteins that interact with such regulatory regions. According to this model, the association of a given RNA-binding protein with a group of mRNAs bearing a specific regulatory sequence provides eukaryotic systems with the plasticity and effectiveness to respond to stimuli of either endogenous or exogenous origin. The present investigation provides evidence that HuR coordinately regulates a subset of genes with critical functions during skeletal myogenesis, thereby lending support to the posttranscriptional operon model.

In the future, it will be of interest to investigate the differentiation process in C2C12 populations exhibiting reduced HuR levels. Our efforts aimed at lowering HuR expression by using antisense HuR-expressing vectors were unsuccessful due to the poor transfectability of these cells and the detrimental effects of low HuR levels on clonal cell growth (reference 40 and data not shown). Use of RNA interference caused all transfected cells to become rounded and detach from the culture dish (not shown). While this observation is in keeping with the notion that HuR contributes to establishing muscle differentiation, further analysis of HuR's role in myogenesis will require the development of efficient approaches to reduce HuR levels, such as mice in which HuR expression can be knocked out in a conditional fashion.

How does the differentiation protocol cause HuR to increase in the cytoplasm of C2C12 cells? Although this question remains to be addressed experimentally, insulin is known to stimulate glucose transport into a number of tissues, including skeletal muscle. In turn, glucose availability causes cellular ATP levels to rise, thereby lowering AMP/ATP ratios. Such high-fuel conditions decrease the activity of the 5′-AMP-activated kinase (AMPK) in the cell. It is important to note that AMPK is the sole enzyme thus far identified as a regulator of the cytoplasmic abundance of HuR; AMPK activation decreases cytoplasmic HuR levels, while AMPK inhibition effectively elevates HuR's cytoplasmic presence (40). The subcellular localization of HuR is believed to be controlled largely through regulated nucleocytoplasmic transport involving the HuR shuttling sequence, HNS (4, 14, 15, 22, 30, 37). Experiments are under way to assess whether interventions to either increase or decrease AMPK can alter the C2C12 differentiation program and to assess the role of HuR in this process.

In summary, we have described a novel function for HuR as a regulator of myogenesis. We postulate that HuR's influence on muscle cell differentiation is intimately linked to its cytoplasmic localization during the early stages of differentiation and its ability to bind to and stabilize mRNAs that encode critical muscle differentiation proteins. Our findings further implicate HuR in the differentiation of muscle cells, underscoring HuR's function within the global adaptive response to changes in extracellular conditions. The present study provides additional support for the role HuR plays as a major regulator of gene expression in response to environmental cues.

ACKNOWLEDGMENTS

We are grateful to J. A. Steitz for kindly providing the 3A2 hybridoma. We thank M. Juhaszova, C. Bailón, and A. Mallabiabarrena for their assistance with confocal microscopy and S. Galbán, W. Wang, I. López de Silanes, K. Mamczarz, and M. Wang for helpful discussions. We appreciate M. González, T. Martínez, E. Serrano, J. L. Martindale, and X. Yang for their technical assistance and our colleague P. J. Morin for critical reading of the manuscript.

The Ministerio de Ciencia y Tecnología (Spain) supported A.F.'s predoctoral and short-term travel fellowships as well as grant SAF2001-2291. U.A. was supported by grant RO1-AI46451.

REFERENCES

1. **Akamatsu, W., et al.** 1999. Mammalian ELAV-like neuronal RNA-binding proteins HuB and HuC promote neuronal development in both the central and the peripheral nervous systems. Proc. Natl. Acad. Sci. USA **96:**9885–9890.
2. **Antic, D., and J. D. Keene.** 1997. Embryonic lethal abnormal visual RNA-binding proteins involved in growth, differentiation and posttranscriptional gene expression. Am. J. Hum. Genet. **61:**273–278.
3. **Antic, D., N. Lu, and J. D. Keene.** 1999. ELAV tumor antigen, Hel-N1, increases translation of neurofilament M mRNA and induces formation of neurites in human teratocarcinoma cells. Genes Dev. **13:**449–461.
4. **Atasoy, U., J. Watson, D. Patel, and J. D. Keene.** 1998. ELAV protein HuA (HuR) can redistribute between nucleus and cytoplasm and is upregulated during serum stimulation and T cell activation. J. Cell Sci. **111:**3145–3156.
5. **Beckel-Mitchener, A. C., A. Miera, R. Keller, and N. I. Perrone-Bizzozero.** 2002. Poly(A) tail length-dependent stabilization of GAP-43 mRNA by the RNA-binding protein HuD. J. Biol. Chem. **277:**27996–28002.
6. **Blattner, C., P. Kannouche, M. Litfin, K. Bender, H. J. Rahmsdorf, J. F. Angulo, and P. Herrlich.** 2000. UV-induced stabilization of c-*fos* and other short-lived mRNAs. Mol. Cell. Biol. **20:**3616–3625.
7. **Brennan, C. M., and J. A. Steitz.** 2001. HuR and mRNA stability. Cell. Mol. Life Sci. **58:**266–277.
8. **Brennan, C. M., I. E. Gallouzi, and J. A. Steitz.** 2000. Protein ligands to HuR modulate its interaction with target mRNAs in vivo. J. Cell Biol. **151:**1–14.
9. **Campos, A. R., D. Grossman, and K. White.** 1985. Mutant alleles at the locus elav in Drosophila melanogaster lead to nervous system defects. A developmental-genetic analysis. J. Neurogenet. **2:**197–218.
10. **Cooper, R. N., S. Tajbakhsh, V. Mouly, G. Cossu, M. Buckingham, and G. S. Butler-Browne.** 1999. In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. J. Cell Sci. **112:**2895–2901.
11. **Chen, C. Y., and A.-B. Shyu.** 1995. AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem. Sci. **20:**465–470.
12. **Dominov, J. A., J. D. Dunn, and J. B. Miller.** 1998. Bcl-2 expression identifies an early stage of myogenesis and promotes clonal expansion of muscle cells. J. Cell Biol. **142:**537–544.
13. **Fan, J., X. Yang, W. Wang, W. H. Wood III, K. G. Becker, and M. Gorospe.** 2002. Global analysis of stress-regulated mRNA turnover using cDNA arrays. Proc. Natl. Acad. Sci. USA **99:**10611–10616.
14. **Fan, X. C., and J. A. Steitz.** 1998. Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. EMBO J. **17:**3448–3460.
15. **Fan, X. C., and J. A. Steitz.** 1998. HNS, a nuclear-cytoplasmic shuttling sequence in HuR. Proc. Natl. Acad. Sci. USA **95:**15293–15298.
16. **Fuchtbauer, E. M., and H. Westphal.** 1992. MyoD and Myogenin are coexpressed in regenerating skeletal muscle of the mouse. Dev. Dynam. **193:**34–39.
17. **Gorospe, M., X. Wang, and N. J. Holbrook.** 1998. p53-dependent elevation of $p21^{Waf1}$ expression by UV light is mediated through mRNA stabilization

and involves a vanadate-sensitive regulatory system. Mol. Cel. Biol. **18:**1400–1407.
18. **Jackman, J., I. Alamo, Jr., and A. J. Fornace, Jr.** 1994. Genotoxic stress confers preferential and coordinate messenger RNA stability on the five gadd genes. Cancer Res. **54:**5656–5662.
19. **Jain, R. G., L. G. Andrews, K. M. McGowan, P. H. Pekala, and J. D. Keene.** 1997. Ectopic expression of Hel-N1, an RNA-binding protein, increases glucose transporter (GLUT1) expression in 3T3-L1 adipocytes. Mol. Cell. Biol. **17:**954–962.
20. **Joseph, B., M. Orlian, and H. Furneaux.** 1998. $p21^{waf1}$ mRNA contains a conserved element in its 3′-untranslated region that is bound by the Elav-like mRNA-stabilizing proteins. J. Biol. Chem. **273:**20511–20516.
21. **Kasashima, K., K. Terashima, K. Yamamoto, E. Sakashita, and H. Sakamoto.** 1999. Cytoplasmic localization is required for the mammalian ELAV-like protein HuD to induce neuronal differentiation. Genes Cells **4:**667–683.
22. **Keene, J. D.** 1999. Why is Hu where? Shuttling of early-response-gene messenger RNA subsets. Proc. Natl. Acad. Sci. USA **96:**5–7.
23. **Keene, J. D., and S. A. Tenenbaum.** 2002. Eukaryotic mRNPs may represent posttranscriptional operons. Mol. Cell **9:**1161–1167.
24. **Loflin, P., C. Y. Chen, and A.-B. Shyu.** 1999. Unraveling a cytoplasmic role for hnRNP D in the in vivo mRNA destabilization directed by the AU-rich element. Genes Dev. **13:**1884–1897.
25. **Ma, W. J., S. Chung, and H. Furneaux.** 1997. The Elav-like proteins bind to AU-rich elements and to the poly(A) tail of mRNA. Nucleic Acids Res. **25:**3564–3569.
26. **Megeney, L. A., B. Kablar, K. Garrett, J. E. Anderson, and M. A. Rudnicki.** 1996. MyoD is required for myogenic stem cell function in adult skeletal muscle. Genes Dev. **10:**1173–1183.
27. **Molkentin, J., and E. N. Olson.** 1996. Defining the regulatory networks for muscle development. Curr. Opin. Gen. Dev. **6:**445–453.
28. **Muscat, G. E., and U. Dressel.** 2000. Not a minute to waste. Nat. Med. **6:**1216–1217.
29. **Olson, E. N.** 1992. Interplay between proliferation and differentiation within the myogenic lineage. Dev. Biol. **154:**261–272.
30. **Peng, S. S., C. Y. Chen, N. Xu, and A.-B. Shyu.** 1998. RNA stabilization by the AU-rich element binding protein HuR, an ELAV protein. EMBO J. **17:**3461–3470.
31. **Ross, J.** 1995. mRNA stability in mammalian cells. Microbiol. Rev. **59:**423–450.
32. **Sachs, A.-B.** 1993. Messenger RNA degradation in eukaryotes. Cell **74:**413–421.
33. **Tenenbaum, S. A., C. C. Carson, P. J. Lager, and J. D. Keene.** 2000. Identifying mRNA subsets in messenger ribonucleoprotein complexes by using cDNA arrays. Proc. Natl. Acad. Sci. USA **97:**14085–14090.
34. **Tenenbaum, S. A., P. J. Lager, C. C. Carson, and J. D. Keene.** 2002. Ribonomics: identifying mRNA subsets in mRNP complexes using antibodies to RNA-binding proteins and genomic arrays. Methods **26:**191–198.
35. **Walsh, K.** 1997. Coordinate regulation of cell cycle and apoptosis during myogenesis. Prog. Cell Cycle Res. **3:**53–58.
36. **Wang, J., and K. Walsh.** 1996. Resistance to apoptosis conferred by Cdk inhibitors during myocyte differentiation. Science **273:**359–361.
37. **Wang, W., H. Furneaux, H. Cheng, M. C. Caldwell, D. Hutter, Y. Liu, N. J. Holbrook, and M. Gorospe.** 2000. HuR regulates p21 mRNA stabilization by UV light. Mol. Cell. Biol. **20:**760–769.
38. **Wang, W., S. Lin, M. C. Caldwell, H. Furneaux, and M. Gorospe.** 2000. HuR regulates cyclin A and cyclin B1 mRNA stability during the cell division cycle. EMBO J. **19:**2340–2350.
39. **Wang, W., X. Yang, V. J. Cristofalo, N. J. Holbrook, and M. Gorospe.** 2001. Loss of HuR is linked to reduced expression of proliferative genes during replicative senescence. Mol. Cell. Biol. **21:**5889–5898.
40. **Wang, W., J. Fan, X. Yang, S. Fürer, I. Lopez de Silanes, C. von Kobbe, J. Guo, S. N. Georas, F. Foufelle, D. G. Hardie, D. Carling, and M. Gorospe.** 2002. AMP-activated kinase regulates cytoplasmic HuR. Mol. Cell. Biol. **22:**3425–3436.
41. **Wisdom, R.** 1999. AP-1: one switch for many signals. Exp. Cell Res. **253:**180–185.
42. **Xu, N., C. Y. Chen, and A.-B. Shyu.** 1997. Modulation of the fate of cytoplasmic mRNA by AU-rich elements: key sequence features controlling mRNA deadenylation and decay. Mol. Cell. Biol. **17:**4611–4621.
43. **Zhang, W., B. J. Wagner, K. Ehrenman, A. W. Schaefer, C. T. DeMaria, D. Crater, K. DeHaven, L. Long, and G. Brewer.** 1993. Purification, characterization, and cDNA cloning of an AU-rich element RNA-binding protein, AUF1. Mol. Cell. Biol. **13:**7652–7665.