INTRODUCTION

Embryonic lethal abnormal vision (ELAV) RNA binding proteins are thought to be involved in cellular growth and differentiation via posttranscriptional regulation (reviewed by Antic and Keene, 1997). ELAV proteins are members of the RNA recognition motif (RRM) superfamily (Query et al., 1989) and each consists of three highly conserved RRMs (Robinow et al., 1988; Szabo et al., 1991; King et al., 1994). Although their exact functions are not known ELAV proteins have been implicated in the stability and translation of early response gene (ERG) messenger RNAs, such as those encoding protooncoproteins and cytokines (Levine et al., 1993; Gao et al., 1994; Jain et al., 1997; Myer et al., 1997). Direct binding of ERG mRNA 3’ untranslated regions (UTR) by ELAV proteins Hel-N1 and Hel-N2 (here termed HuB) was demonstrated following in vitro selection from combinatorial RNA libraries (Levine et al., 1993; King et al., 1994; Gao et al., 1994). To date, four classes of ELAV proteins have been described and all appear to have similar properties of binding to AU-rich elements (ARE) in these 3’UTRs (reviewed by Antic and Keene, 1997). However, differences in tissue distribution among the four classes and their temporal appearance during embryonic development (Good, 1995) suggests that protein interaction signals outside of the RRMs could endow each member with unique functional properties (King et al., 1994; Gao and Keene, 1996; Antic and Keene, 1998).

Although none of the mammalian ELAV proteins localizes to a single compartment, the tissue-specific forms HuB, HuC and HuD, are predominantly cytoplasmic in cultured NIH-3T3 cells, but is localized in the cytoplasm during early G1 of the cell cycle. Therefore, much like the primarily cytoplasmic HuB, HuA becomes temporally localized in the cytoplasm where it can potentially regulate the stability or translation of bound mRNAs. Moreover, we report that stimulation of mouse spleen cells using either mitogenic or sub-mitogenic levels of anti-CD3/CD28 resulted in a dramatic increase in the level of HuA. Uprogulation of HuA corresponds to previously documented increases in cytokine expression which are due to increased mRNA stability following T cell activation. Consistent with these findings, HuA was down regulated in quiescent cells and upregulated in 3T3 cells following serum stimulation. The increase of murine HuA during the cell cycle closely resembles that of cyclin B1 which peaks in G2/M. Together with our earlier studies, these data indicate that mammalian ELAV proteins function during cell growth and differentiation due in part to their effects on posttranscriptional stability and translation of multiple growth regulatory mRNAs. This supports the hypothesis that ELAV proteins can function as transacting factors which affect a default pathway of mRNA degradation involved in the expression of growth regulatory proteins.

Key words: RNA binding protein, RRM, AU-rich elements, 3’ untranslated region, RNA stability, Translation, Cytokine, Paraneoplastic disease
with transcription inhibitors actinomycin D (ActD), and 5,6-dichloro-β-d-ribofuranosyl benzimidazole (DRB) and reimport into the nucleus upon washout of DRB. Moreover, we find that in untreated NIH-3T3 cells, endogenous HuA can be found predominantly in the cytoplasm during early G1 of the cell cycle.

Our previous demonstration that ectopic expression of the human ELAV protein HuB (Hel-N1) can result in increased stability and translation of the ERG mRNA encoding endogenous GLUT1 protein (Jain et al., 1997), as well as, endogenous c-myc mRNA (unpublished data) was unexpected since ARE-binding proteins were assumed to help destabilize target mRNAs (Vakalopoulou et al., 1991). These observations were confirmed by the finding that vascular endothelial growth factor (VEGF) mRNA is stabilized during hypoxia following ectopic HuA (HuR) expression (Levy et al., 1998). Accumulated data suggest that ELAV proteins may function as transacting factors which modulate a default pathway of ARE-mediated mRNA degradation.

It is well established that cytokine mRNAs are stabilized following proliferation and artificial stimulation of T cells (Lindsten et al., 1989). Bohjanen et al. (1991) demonstrated following T cell activation that an inducible protein, AU-B, cross-links to the AREs of RNAs encoding cytokines such as IL-2, TNF-alpha and GM-CSF. However, this protein has not been identified. We have investigated the expression of ELAV proteins, in particular HuA, during proliferation and T cell activation to determine whether its levels increase during periods following activation and subsequent cytokine expression. We report that following costimulation of murine T cells by CD3 and CD28 activation, levels of mHuA increased significantly, as one might expect of a transacting factor(s) involved in ARE-mediated stabilization of cytokine mRNAs. Since activation of T cells results in their recruitment from quiescence into the cell cycle, we examined HuA expression in NIH-3T3 cells. We report that rapidly growing 3T3 cells have increased levels of the mHuA protein, while quiescent cells have lower levels. The kinetics of mHuA expression were found to be similar to those of cyclin B1 expression, which is maximally expressed during G2/M, but then diminishes.

In total, these results are compatible with our previous findings that ELAV proteins, as exemplified by HuB, participate in the stabilization of ERG mRNAs presumably via binding to ARE 3’UTR sequences in the cytoplasm. These findings suggest a role for the ELAV protein, HuA, in cellular proliferation. Furthermore, by its increased levels in the cytoplasm during and following cell division, mHuA may have a temporal role in mediating expression of mRNAs via their stabilization or translation. These findings have important implications for the regulation of cell proliferation during homeostatic growth, as well as during immunoregulation.

MATERIALS AND METHODS

Cloning of HuA forms of ELAV from mouse brain, spleen and testis

The gene for mHuA (accession number U65735) was cloned by using 5’ and 3’ RACE (rapid amplification of cDNA ends) techniques using the following Clontech products: Marathon-Ready murine spleen and brain cDNA libraries, Advantage KlenTaq Polymerase Mix (Clontech, Palo Alto, CA). Gene specific HuA primers (GSP1 5’AGAACCTGAATCTCTGTGCTG, position 307; GSP2 (5’CTAGACGGCGATGGTACAQCT, position 261) were designed from the partial length murine HuA sequence (kind gift of Peter Good, accession number U17595). For 5’ RACE, two nested PCR were performed following the recommendations in the Clontech manual. For the first reaction, forward primer AP1 and reverse primer GSP1 were used. PCR was performed using ‘touchdown’ parameters: 95°C for 2 minutes, followed by 25 cycles of: 100°C for 10 seconds, 68°C for 1 minute; then 20 cycles of 100°C for 2 seconds, 70°C for 1 minute; then 20 cycles of 100°C for 2 seconds, 68°C for 1 minute. Two additional rounds of nested PCR were performed as per the Clontech protocol using the following primers: forward primer AP2; reverse primer GSP2. The final round of nested PCR used AP2 and 5’CCACTGACGCTGGGGCGAGCATA, position 422, human sequence HuR, accession U38175). Nested 3’RACE was performed using published Clontech touchdown parameters. For the first reaction, the following primers were used: reverse primer AP1 and forward primer GSP1 5’CGTCTAGACGGAGAG. Position 113. For nested reaction: reverse primer AP2; forward primer GSP2 5’TGTCCCTCGTGGCTGTTAGTGAAG (position 227). PCR products were electrophoresed, transferred to Nytran membranes and probed with 32P-end labeled HuA primers. Positive bands were subcloned into the pNOTA vector (5Prime-3Prime) and sequenced in both directions with universal M13 and HuA specific primers. Simultaneously, full-length clones were obtained from the Marathon brain and spleen cDNA libraries using the following primers (derived from human clone, HuR (HuA), accession U38175) forward primer, 5’ ACAATGCTAATAGTATGGA (position 116) and reverse primer, 5’ GACGCCAGTTATTTATTCGA (position 1,106). The reactions were cycled using the published Clontech protocol. A solitary band of the expected size (approximately 1 kb), was confirmed by Southern analysis to be HuA specific from both brain and spleen libraries. The full-length 1 kb PCR products were subcloned into pNOTA (5Prime-3Prime, Boulder, CO) and sequenced in both directions using the ABI method and universal M13 and HuA specific primers. The sequences obtained from the 5’, 3’ RACE reactions were identical to those obtained from the full-length clones generated using human primers. Additionally, putative full-length clones were obtained from mouse testis cDNA (Clontech, Palo Alto, CA) using the published Clontech protocol and the human specific primers described above. 5 μl of the PCR reaction (representing 10% of the total volume) was electrophoresed, blotted onto Nytran and probed with an internal HuA primer. A prominent and single 1 kb band was identical to the full-length clones obtained from mouse brain and spleen cDNA (data not shown).

Derivation of mHuB probe

Gene specific primers (forward primer 5’ GGCGATCACACTCTGAATTGG and reverse primers 5’ GTACATCAGGACAGCTGTCT) were designed from a partial length rat ELAV clone which had been used to clone the human ELAV gene, Hel-N1 (King et al., 1994). Reverse transcriptase-PCR was performed on mouse brain poly A+ (Clontech) using published Perkin Elmer protocols, the Gene Amp RNA PCR kit (Norwalk, CT) and the rat primers above. Gel electrophoresis of the final PCR product revealed a solitary band of the expected size which was subcloned into pNOTA vector (5Prime-3Prime) and confirmed by sequencing to be the murine homolog of rat HuB cDNA. The plasmid was digested with BamHI and the ds cDNA for mouse HuB (Mel-N1) cut out of the gel and used in random prime labeling with [32P]dCTP using the random nonomer kit from Amersham (LaJolla, CA).

Northern blot analysis

Northern blots of mouse and human tissues, as well as human tumor
lines (Clontech) were hybridized with a PCR-generated 358 bp HuA probe or with the mHuB probe described above. The PCR product was subcloned into the pNOTA vector, sequenced to verify its identity and then used as a template for further reactions. The double-stranded DNA was labeled by the random nonomer method (Stratagene) and the northern blots were hybridized with probes using ExpressHyb hybridization solution (Clontech) and subjected to autoradiography for various times. All northern blots contained 2 µg of poly(A)+ RNA per lane and have been quality tested using actin DNA probe as control to ensure equivalent amounts of RNA loaded per lane.

**Cell lines**

Cell lines were obtained from ATCC except where described and cultured under ATCC recommended conditions unless indicated otherwise. HFF (human foreskin fibroblasts) were obtained from Dr Ross McKinney (Duke University Medical Center).

**Cell cycle synchronization protocols**

**Serum starvation of NIH-3T3 cells**

NIH-3T3 cells growing in DMEM+10% bovine calf serum (BCS) were trypsinized, split 1:10 and seeded into P150 Petri dishes. After overnight growth they were washed twice in DMEM and then starved for 48 hours in DMEM+0.25% BCS. After serum starvation, the medium was aspirated and fresh DMEM+10% BCS was added. Cells were harvested at various time points (see Results) by tryspinization, washed with ice-cold PBS and then lysed using triple-detergent lysis buffer (see below). At each time point, an aliquot of cells was stained with propidium iodide (PI) and analyzed by FACS to confirm stages of cell cycle. Typically, 86-95% of the cells were in G0 after 48 hours of serum starvation.

**Hydroxyurea block of NIH-3T3 cells**

Exponentially growing cells in DMEM+10% BCS were serum starved as above for 32-36 hours. These plates were aspirated, washed two times with DMEM and treated with DMEM+10% BCS containing hydroxyurea (final concentration, 2 mM; Sigma) for 21 hours. The hydroxyurea was removed by washing the cells twice with DMEM. DMEM+10% BCS was added back for varying times. Cells were harvested and lysed as described above. Aliquots of unlysed cells were assayed by FACS PI staining to confirm stages of cell cycle. The vast majority of the cells were in G1/S after hydroxyurea treatment.

**Activation of murine splenocytes**

Single-cell suspensions of freshly teased and Ficoll-Hypaque (Fico-Lite, Atlanta Biologics, Atlanta, GA) isolated splenocytes from C57B6 mice were prepared. These unstimulated murine splenocytes were suspended at 2x10^6/ml with different concentrations of anti-CD3 (145-2C11 from Jeff Bluestone, Chicago, IL) and/or anti-CD28 (37.51 from J. P. Allison, Berkeley, CA) monoclonals. Anti-CD3 at 1:80 is mitogenic and gives 100% response on [3H]thymidine uptake: at 1:6,400 it is sub-mitogenic and gives a thymidine response of less than 10% of maximal. Anti-CD28 at 1:800 does not stimulate by itself but synergizes with anti-CD3 at 1:6,400 to give maximum response (Lee et al., 1998). Aliquots of cells were removed at various points, washed and lysed using triple detergent buffer and quantitated using Bradford analysis. Equal amounts of protein were electrophoresed on 12% SDS gel, transferred onto nitrocellulose and blotted with antisera to mHuA or tubulin.

**Antibody production, western blot analysis and quantitation**

cDNA for HuA was cloned into plasmid pGexKG for overexpression of the recombinant GST-tagged protein. Thrombin was used in a partial digest to remove the GST tag and the band corresponding to full length HuA cut out of a SDS-PAGE gel and used for polyclonal rabbit antibody production (Cocalico, Inc.). For immunofluorescence staining the antibody was further purified over an affinity column (ImmunoLink® Plus, Pierce) of GST-HuA and eluted with 0.1 M glycine, pH 2.6, and neutralized with Tris-HCl, pH 9.5. Western blot analysis of cell lysates demonstrated that a band of the expected size for HuA was removed from the antibody flow-through and presents as a solid band in the predicted fractions eluted from the column. By western blot analysis, HuA antisera did not cross-react with endogenous forms of other neuronal specific ELAV family members. However, prior to affinity purification we consistently observed a band of approximately 28 kDa which may represent a degradation product, but also could be an additional ELAV family member.

Whole cell lysates were prepared at 10^6 cells per ml in triple lysis buffer of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Nonidet P40, 0.5% sodium deoxycholate and protease inhibitors (1 mM PMSF, 10 µg/ml aprotinin, 1 µg/ml leupetin, 1 µg/ml pepstatin from Sigma) and sonicated for 10 seconds three times. This method solubilized all cellular proteins. Protein concentrations were determined by Bradford assay and 20-40 µg loaded per lane in 1x Laemmli buffer. Following transfer to nitrocellulose and blocking with 5% non-fat milk in TBS-Tween, primary antibody incubations were carried out in TBS-Tween for 2 hours at room temperature or overnight at 4°C. The secondary antibodies conjugated to horseradish-peroxidase (HRP) (Amersham) were incubated at room temperature for 30 minutes and the blots were developed using the ECL system according to the manufacturer’s directions (Amersham). Anti-tubulin murine monoclonal antibody was used at a dilution of 1:2,000 (Amersham).

Western blots were scanned densitometrically and HuA and tubulin signals in the linear range were quantitated using ImageQuant (Molecular Dynamics). To calculate relative HuA signal in densitometric units, the values were normalized to the tubulin signals. Limiting dilution western blots for HuA and tubulin were done using ECL and [125I]-labelled Protein A. The [125I]-labelled blots were scanned by phosphorimager and quantitated using ImageQuant. Both methods yielded results consistent with one another.

**Immunofluorescence staining**

3T3 cells were grown in chamber slides (Nunc). Cells were washed in PBS and fixed in paraformaldehyde in poly-lysine and stained as described previously (Gao and Keene, 1996). A further fixation in 50% v/v acetone/methanol for 1 minute was found to increase the intensity of staining for HuA. Affinity purified antibody was used diluted 1:1 in blocking buffer. For identification of 3T3 cells in S phase combined with HuA staining, bromodeoxyuridine (BrdU) was included in the culture medium for one hour as described by DeGregori et al. (1995). DAPI was added at 2 mg/ml for 2-5 minutes at room temperature, following immunostaining protocols where described. There was no staining when anti-HuA primary antibody was omitted. Secondary antibodies used included a donkey anti-rabbit-Texas Red (Jackson ImmunoResearch, Inc.) and an FITC-conjugated mouse monoclonal against BrdU (Amersham). Monoclonal antibody 4B10 to hnRNP A1 was provided by Gideon Dreyfuss (University of Pennsylvania).

**Cell fractionation**

3T3 cells were separated into fractions based on the Weinberg and Penman (1968) method. Cells were resuspended at 10^7/ml in a hypotonic buffer of 10 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1.5 mM MgCl2 with protease inhibitors and allowed to swell for 5 minutes. Lysis was achieved by addition of 0.5% Nonidet NP40 and nuclei were pelleted at 1,000 g for 2 minutes. The supernatant was removed and stored as cytoplasmic fraction C1. The outer nuclear membrane (and attached material) was removed by washing nuclei in the above buffer containing 1% NP40 and 0.5% sodium deoxycholate. The solubilized supernatant after centrifugation at 1,000 g for 2 minutes was designated C2. The nuclear pellet was sonicated in triple lysis buffer and the fraction termed N. SDS-PAGE gels were loaded with equal cell equivalents per lane. Transcription was inhibited by treating
cells with 5 μg/ml actinomycin D for up to 3 hours. 5,6-dichloro-β-D-ribofuranosyl benzimidazole (DRB) (Sigma) was also used to inhibit transcription at 100 μM for 3 hours and was reversed by washing out the drug following a further incubation in medium for 3 hours.

RESULTS

Cloning and expression of mHuA

We derived full length cDNAs (from brain and spleen) encoding the mouse ELAV protein corresponding to Xenopus elrA using the partial mouse clone derived by Dr Peter Good at the National Institutes of Health (Good, 1995). Expression of elrA and its human counterpart HuR (HuA) has been reported at the mRNA level (Good, 1995; Ma et al., 1996; Okano and Darnell, 1997). elrA has also been detected in Xenopus oocytes by immunoblotting (Wu et al., 1997). We investigated expression of HuA protein in a variety of mammalian cell types using high titer polyclonal rabbit antisera prepared against recombinant mHuA and found relative differences between levels in transformed (Fig. 1A and B, lanes 3 and 4) and nontransformed (Fig. 1B, lanes 1 and 2) cell lines. Human foreskin fibroblasts and embryonic kidney fibroblasts were chosen to illustrate extreme examples (Fig. 1B, lanes 1 and 4, respectively). In all cases examined, a discrete protein of the expected size of 34 kDa was detected using affinity purified mouse HuA antiserum. These and other data not shown demonstrated that the antiserum recognized mouse HuA (mHuA) and human HuA equally well. Since there were differences in relative protein expression levels among cell types, it was important to examine mHuA mRNA expression in a variety of cell lines.

Brain and testes express unique forms of HuA mRNA

By northern blot analysis we assessed the expression of both HuA and HuB in a variety of mouse tissues. As shown in Fig. 2 quantitative and qualitative differences were also observed among the tissues examined. In the majority of tissues HuA was expressed as a single unique mRNA of approximately 2.6 kb (Fig. 2A, upper panel) that varied in relative intensity among cell lines. However, in brain and testes the 2.6 kb band was not expressed or was barely detectable, while new and unique mRNA species of 6.6 kb and 1.8 kb appeared. For comparison upon reprobing, the same northern blot demonstrated the expression of HuB mRNAs of approximately 4.5 kb in brain and testes (Fig. 2A, lower panel). As expected, other tissues and cell lines that expressed HuA did not have high levels of mHuB mRNA under the same conditions of blotting. These findings were confirmed further by examining a variety of human tissues and tumors for expression of HuA mRNAs (Fig. 2B, both upper and lower). The mRNAs detected in these human tissues corresponded precisely with those observed with the mouse tissues including brain and testes. Thus, it is clear that HuA represents a generic or ubiquitous member of the mammalian ELAV family which is detectable in every tissue of mouse and human examined. Previously Okano and Darnell (1997) reported the presence of a 1.8 kb band of HuA mRNA in testes but did not report a higher sized mRNA transcript in brain. Ma et al. (1996) reported only a single sized band using RT-PCR, which may not have been able to detect all potential transcripts.

Full length mHuA clones from both brain and spleen had identical sequences in the open reading frame (data not shown). Additionally, full-length clones obtained from mouse testis cDNA libraries by PCR and Southern blotting also were identical in size to those obtained from mouse brain and spleen (data not shown). At the protein level, different sized bands were not observed in whole mouse brain extracts using our polyclonal antibody raised against recombinant HuA. Indeed, we have consistently observed a band of approximately 34 kDa which corresponds to mHuA. Hence, it seems plausible that the differences in transcript size in mouse brain, spleen and testis are due to differences in the untranslated regions of the mRNAs. Okano and Darnell (1997) have reported heterogeneity in the 3'UTRs of mHuA. However, we have not ruled out differential splicing or the existence of isoforms of the HuA mRNA in these tissues. We conclude that there are HuA transcripts in both mouse brain and spleen encoding identical full-length open reading frames of the HuA protein.

Expression of HuA and mHuB during mouse embryogenesis

Given the potential role of ELAV proteins in differentiation (Robinow and White, 1991; Wu et al., 1998; reviewed by Antic and Keene, 1997), we decided to probe northern blots representing different stages of murine development with the mHuA probe. There was a single band of the expected size (2.6 kb) on days 11, 15 and 17 (Fig. 2C, upper). However, there was no mHuB transcript detected at day 7 (Fig. 2C, lower panel, lane 1) which is consistent with the fact that the murine nervous system begins to develop after day eight. Hence mHuB is expressed about the time of the earliest development of neurons in the murine central nervous system and is consistent with the time of appearance of ELAV in Drosophila (Robinow
et al., 1988). In contrast to the developmental expression of mHuB, however, mHuA appeared to be constitutively expressed throughout development as determined at day 7 (lane 1), the earliest time examined. Furthermore, mHuA continued to be expressed throughout intruterine development as would be expected of a constitutive generic cell protein. The fact that the 6.6 kb and 1.8 kb transcripts were not detected in developmental northern blots is probably due to the vanishingly small amounts of the tissues from brain and testes represented during these early stages of development. We conclude that mHuA is expressed at early stages of murine development, which is consistent with its being generic and not tissue-specific.

**Expression of mHuA during cell cycle progression in NIH-3T3 cells**

Given the apparent variation in expression of mHuA protein (Fig. 1) and RNA (Fig. 2) among the cell lines examined, we wondered whether there may be differences in protein levels at various proliferative stages for any given cell type. Therefore, cell cycle synchronization experiments were carried out using 3T3 with two established protocols: either serum starvation or hydroxyurea block, followed in both cases by serum restimulation. Levels of mHuA were monitored by western blotting using affinity purified antiserum. As shown in Fig. 3A, starved cells in G0 showed very low levels of protein (lane 2), but the levels gradually increased after serum was added back to cells, peaking at 18 hours post stimulation (arrow, lane 5). HuA levels as measured in arbitrary densitometric units increased approximately 2-fold during these experiments. FACS staining of these same cells with propidium iodide (PI) (not shown), confirmed that serum starvation placed the cells in G0 and upon serum re-stimulation they progressed through the cell cycle normally. The 18 hour time point (lane 5), during which mHuA levels reached maximum, represented G2/M with 41% of the cells in G2/M by FACS PI staining (data not shown). We reproducibly observed an inexplicable slight decrease in mHuA at 24 hours post serum addition (lane 8). Nonsynchronous 3T3 cells when harvested during logarithmic growth showed higher levels of mHuA (lanes 1 and 9). Consistent with these findings, nonsynchronous cells that reached 100% confluence showed lower levels of mHuA (lane 10). Therefore, although the degree of synchrony was not optimal when using the serum starvation method, a definitive correlation between the levels of mHuA and certain stages of proliferation was evident.

We further synchronized 3T3 cells using the more efficient method of hydroxyurea block which arrests cells in G1/S (Jong et al., 1995) followed by reentry into the cell cycle after readdition of serum and removal of drug. Throughout these experiments, FACS PI staining was used to monitor the stages of cell cycle (data not shown). As shown in Fig. 3B, the levels of mHuA decreased from time 0 (G1/S, lane 1) to 3 hours post release (lane 2), increased progressively as proliferation ensued reaching a peak at 8 hours (arrow, lane 4) and then progressively decreased. The 8 hour time point shown in lane
shown in Fig. 3A, serum starved 3T3 cells (Fig. 3B, lane 7) cyclin B1 levels varied by about 5-fold. Consistent with results in arbitrary densitometric units varied up to 5-fold, whereas the experiment shown in Fig. 3B, the levels of mHuA as measured is known to peak at G2/M in the mammalian cell cycle (Maity et al., 1995). Interestingly, mHuA expression paralleled cyclin B1 expression with the peak occurring around G2/M and the approximate the expression of cyclin B1.

The higher level of mHuA found following hydroxyurea blockage (Fig. 3B, lane 1) is consistent with the fact that blockage occurs at the G1/S boundary. However, the significance of the reproducible dip in HuA expression following serum re-stimulation is presently unknown (Fig. 3B, lane 2). Given these observations which indicate that levels of mHuA vary with the state of proliferation, we decided to examine the response of mHuA during activation of immune cell proliferation.

**mHuA expression is upregulated during T cell activation in mouse splenocytes**

One of the established examples in which post transcriptional regulation at the level of mRNA stability is known to affect cytokine protein expression is that of T cell activation via CD3 and CD28 signaling (June et al., 1987; Lindsten et al., 1989; Thompson et al., 1989). Since the ELA V family members, including mHuA, have been shown to bind to the 3'UTRs of cytokine mRNAs and mHuA is the first known ELA V family member to be expressed in cells of lymphoid lineage (Figs 1 and 2; spleen, thymus, MOLT-4, a T cell line, Burkitt's lymphoma and Namalwa, a type of Burkitt's lymphoma) we investigated whether mHuA levels were also altered during activation of T cells. We examined mouse spleen cells for levels of mHuA following standard methods of activation (see Materials and Methods) with either mitogenic levels of anti-CD3 or with sub-mitogenic, synergistic levels of anti-CD3 plus anti-CD28 (Lindsten et al., 1989). As shown in Fig. 3C, lane 1, mHuA was barely detectable in untreated cells, which is consistent with the quiescent (G0) state. Interestingly, unstimulated human peripheral blood lymphocytes have nearly undetectable levels of HuA mRNA (Fig. 2B, lane 8). Following stimulation with mitogenic levels of anti-CD3, an increase in mHuA was evident by as early as 24 hours (lane 4), but no change occurred upon addition of anti-CD28 alone (lane 5) or in untreated controls (lane 2). An increase in mHuA expression was also observed at day 1 when co-stimulating with sub-mitogenic doses of anti-CD3 plus anti-CD28 (lane 6). By day 2 in the presence of anti-CD3 (lane 7) the level of mHuA was higher as compared to unstimulated controls. Therefore, consistent with data shown above using 3T3 cells (Fig. 3A), mitogenic stimulation of T cells also upregulated levels of HuA up to 12-fold as measured in arbitrary densitometric units.

Moreover, when these lymphoid cells were stimulated with sub-mitogenic levels of both anti-CD3 and anti-CD28, for two days, there was approximately a 12-fold increase in the level of mHuA (lane 9) as compared with either unstimulated or anti-CD28 controls (lanes 2 and 8, respectively). Furthermore, by day 2 following costimulation using both antibodies (lane 9) the levels of mHuA reached those consistently observed in

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**Fig. 3.** Western blot analysis showing expression of mouse HuA in NIH-3T3 (A and B) or mouse splenocytes (C) following treatments to activate proliferation. (A) Mouse 3T3 cells following serum starvation (0.25%) and reactivation by serum addition (10%). Lanes: 1, exponentially growing, unstarved cells; 2, serum starved for 48 hours (G0); lanes 3-8: post serum addition; 3, 10 minutes; 4, 6 hours; 5, 18 hours; 6, 20 hours; 7, 22 hours; 8, 24 hours; 9, exponentially growing 3T3 from another experiment; 10, confluent cells (G0). B: mouse 3T3 cells following growth blockage with hydroxyurea and subsequent release. Lanes: 1, t=0, no serum added; lanes 2-6: post serum addition; 2, 3 hours; 3, 6 hours; 4, 8 hours; 5, 10 hours; 6, 12 hours; 7, starved cells with no hydroxyurea treatment; 8, untreated cells; 9, positive control, exponentially growing 3T3 cells. G2/M is approximately marked by arrows. C: mouse spleen cells treated with anti-CD3 and/or anti-CD28 to activate T cells. Lanes: 1, cells at day 0; 2, cells unstimulated at day 1; 3, cells unstimulated at day 2; lanes 4-9, stimulated with either or both antibodies; 4, anti-CD3 (1:80) at day 1; 5, anti-CD28 (1:800) at day 1; 6 anti-CD3 (1:6400)+anti-CD28 (1:800) at day 1; 7 anti-CD3 (1:80) at day 2; 8 anti-CD28 (1:800) at day 2; 9 anti-CD3 (1:6400)+anti-CD28 (1:800) at day 2; 10, control 3T3 cells in log phase of growth. In each blot, equivalent amounts of protein were loaded in each lane and tubulin was probed on the same blots as control. Results shown are representative of three separate experiments.

4 represents G2/M as assayed by FACS PI staining and by monitoring maximal cyclin B1 expression (Fig. 3B). Cyclin B1 is known to peak at G2/M in the mammalian cell cycle (Maity et al., 1995). Interestingly, mHuA expression paralleled cyclin B1 expression with the peak occurring around G2/M and the lowest levels in G0/G1. In the hydroxyurea block-release experiment shown in Fig. 3B, the levels of mHuA as measured in arbitrary densitometric units varied up to 5-fold, whereas the cyclin B1 levels varied by about 5-fold. Consistent with results shown in Fig. 3A, serum starved 3T3 cells (Fig. 3B, lane 7) and exponentially growing cells (Fig. 3B, lane 9) showed levels of mHuA that correlated with the proliferative state, while the higher levels were always observed in actively growing cells. The cells in lane 8 were untreated (negative control) and completely confluent. Thus, the levels of HuA in these untreated cells were very similar to those observed with starved cells (lane 7). We conclude that levels of the ubiquitously expressed ELAV protein, mHuA, are higher during cell proliferation reaching a peak around G2/M and they appear to
all of our exponentially-growing 3T3 cells (lane 10). Stimulation with only anti-CD3 at sub-mitogenic levels (1:6,400) showed negligible HuA upregulation (data not shown). These data demonstrate that stimulation of quiescent T cells using antibodies to CD3 and CD28 surface receptors in mitogenic or submitogenic levels resulted in dramatic increases in the expression levels of the ELAV protein, mHuA. HuA levels increase following stimulation of either NIH-3T3 cells or murine splenocytes. The greatest increases are seen when T cells are stimulated. This may be due to the fact that nearly 100% of unstimulated murine splenocytes are in G0, whereas the previously employed methods used to block NIH-3T3 cells never block 100% of the cells.

The sub-mitogenic response used in the experiment of Fig. 3C corresponds with well documented increases in cytokine mRNA stability following costimulation of T cells (June et al., 1987; Lindsten et al., 1989). Furthermore, it is consistent with our findings that the ELAV protein, HuB (Hel-N1) results in stabilization of target mRNAs following transfection of 3T3L1 cells (Jain et al., 1997; Antic and Keene, 1997; unpublished data) and that ectopic HuA expression stabilizes VEGF mRNA (Levy et al., 1998).

The correlation shown above between increased levels of mHuA, previously documented increases in cytokine mRNA stabilization and T cell activation may appear to be inconsistent with the reported localization of HuR to the nucleus (Vakalopoulou et al., 1991; Myer et al., 1997). However, as shown below, mHuA can be detected also in the cytoplasm of NIH-3T3 cells in low amounts and has a predominant cytoplasmic presence at a particular stage of the cell cycle.

**Cellular localization of mHuA**

Given the potential regulation of mHuA during cell proliferation, as shown above, it is believed that the stability and translation of ERG polyadenylated mRNA most likely takes place in the cytoplasm (see Richter, 1996, and articles therein). The putative localization of mHuA to the nucleus was based previously upon biochemical fractionation and binding of cell extracts to ERG RNAs in vitro (Vakalopoulou et al., 1991; Myer et al., 1997). Since antibodies to mHuA have not been available to assess its expression in mammalian cells, we used our antisera against mHuA to examine its intracellular location. Our affinity purified antisera reacts only with the ubiquitous protein HuA and does not recognize any other cell proteins or other ELAV proteins based upon western blotting of many cells and tissues. As expected, examination of 3T3 cells by indirect fluorescence using this antisera revealed predominantly nuclear staining (Fig. 4A). However, when a large number of fields were examined, separating daughter cells or recently separated cells were observed in which cytoplasmic staining was dramatically increased (Fig. 4C and D). The rarity of these cells appears to reflect the short time period during which cells are in this stage of the cell cycle. Interestingly, these cells had a strong pattern very similar to that reported previously with medulloblastoma cells expressing the HuB ELAV proteins Hel-N1 and Hel-N2 (Gao and Keene, 1996). This suggests that at a distinct period during the cell cycle, presumably during early G1, mHuA protein is located in the cytoplasm and later returns to the nucleus. Double staining with anti-BrdU-FITC to track the S phase of the cell cycle clearly demonstrated mHuA was predominantly nuclear during periods of DNA synthesis (Fig. 5A and B). The recently divided cells in Fig. 5A and B (arrow), which displayed cytoplasmic mHuA staining did not incorporate BrdU and hence were not in S phase. Fig. 5C and D, as well as Fig. 5E and F, indicate cells which were involved in various stages of mitosis as detected by DAPI staining. Fig. 5D (arrow) outlines the condensed chromosomes and displays diffuse HuA staining (Fig. 5C) which may be a result of nuclear membrane breakdown during mitosis. Fig. 5E and F demonstrate the predominantly cytoplasmic staining of HuA at times when the DAPI staining shows that new daughter nuclei have formed.

Staining of endogenous HuA suggested that the protein could redistribute to the cytoplasm in a cell cycle dependent manner. We further investigated HuA localization after inhibition of transcription which, originally shown for hnRNPA1, can cause redistribution of some nuclear proteins into the cytoplasm (Pinol-Roma and Dreyfuss, 1991). A further rationale for this experiment comes from Katz et al. (1994) who described a UV-crosslinked ARE binding protein similar in size (34 kDa) to mHuA that accumulated in the cytoplasm of T cells following actinomycin D (ActD) treatment. Upon addition of ActD (5 μg/ml, 3 hours) to NIH-3T3 cells, mHuA dramatically increased in the cytoplasm leaving in some cells the nucleus almost devoid of staining (Fig. 4B). 5,6-dichloro-β-d-ribofuranosyl benzimidazole (DRB), which inhibits transcription by a different mechanism than Act D also caused redistribution of HuA to the cytoplasm (Fig. 6A). Following washout of DRB, HuA returned to its predominantly nuclear distribution (Fig. 6D). This experiment demonstrated both the export of HuA into the cytoplasm and its reimport into the nucleus. The effect of DRB (Fig. 6B) and DRB washout (Fig. 6E) on hnRNPA1 staining is also shown. In comparison to HuA, the proportion of hnRNPA1 that leaves the nucleus during treatment with inhibitor was small.

To further confirm these observations the expression levels of HuA in the different compartments were investigated by fractionation of Act D treated 3T3 cells and western blotting (Fig. 7). Subcellular fractionation was performed using the method outlined by Weinberg and Penman (1968) which allows separation of the cytoplasm into soluble (C1) and outer nuclear membrane-associated (C2) components. The integrity of the fractionation before and after ActD treatment was confirmed by monitoring of nuclear antigens, U1 70K and Sm (data not shown). In untreated cells (control), HuA was predominantly nuclear (N) but some soluble cytoplasmic (C1) and detergent extractable protein (C2) was also evident. ActD treatment resulted in a significant increase in the mHuA cytoplasmic signal and a corresponding decrease in the nuclear signal. These data demonstrate a dramatic transition of the ubiquitously-expressed ELAV protein HuA as compared with the other ELAV family members which appear to be predominantly cytoplasmic.

Although small amounts of HuA can be detected in the cytoplasm of cells at all phases of the cell cycle, it is highly abundant during a specific phase of the cell cycle, and thus has the opportunity to interact with ERG RNAs in the cytoplasm. This observation does not preclude the possibility that a basal level of movement in and out of the cytoplasm is occurring continually, even though the predominant localization of mHuA appears nuclear.
Fig. 4. Indirect immunofluorescence showing the localization of mHuA in mouse 3T3 cells. (A) Predominantly nuclear localization of mHuA in exponentially growing, nonsynchronized cells; B, cytoplasmic localization of mHuA following treatment of nonsynchronized 3T3 cells for 3 hours with 5 μg/ml actinomycin D; (C and D) same as A, but showing fields in which mHuA is localized in the cytoplasm of recently separated 3T3. HuA was visualized using Texas Red.

Fig. 5. Double staining of 3T3 cells with mHuA antisera and anti-BrdU reveals nuclear localization of mHuA during S phase of cell cycle and cytoplasmic distribution after mitosis. (A and B) Cytoplasmic mHuA distribution in recently dividing cells (arrow). Cells in B which stain with anti-BrdU (FITC) are in S phase and co-localize in the nucleus with mHuA (Texas Red). (C and D) A cell with condensed chromosomes about to undergo mitosis (arrow). (E and F) Cells which have recently completed mitosis. Blue DAPI staining clearly outlines nuclear material.
**DISCUSSION**

ELAV proteins have all been found to bind to AU-rich sequences present in the 3'UTRs of mRNAs encoding protooncogenes and cytokines. While HuA appears to have the same in vitro RNA binding specificity as HuB, HuC and HuD, it shows differences in expression and intracellular localization which may indicate distinct functions. In this paper we have investigated the level of expression, tissue specificity and intracellular localization of HuA. We prepared rabbit antiserum against recombinant HuA that does not recognize the neuronal ELA V proteins, thus allowing specific detection of endogenous HuA and hnRNPA1 returning to their original nuclear staining (D and E). Double staining for HuA and hnRNPA1 is shown in both cases in C (DRB treatment) and F (DRB washout).

**Intracellular localization of the HuA ELAV protein**

Sequence comparisons of the mammalian ELAV proteins shows that there is strong identity in the RRM s which is consistent with their RNA binding activities being virtually indistinguishable (Query et al., 1989; Levine et al., 1993; King et al., 1994; Gao et al., 1994; Ma et al., 1996; Myer et al., 1997). While all ELAV proteins can bind the same RNAs in vitro, apparent differences in the intracellular localization of ELAV
proteins has raised the question of how they might find access to the same mRNA targets in vivo. For example, the HuB proteins, Hel-N1 and Hel-N2, are predominantly cytoplasmic with some nuclear staining (Gao and Keene, 1996). Endogenous HuA, on the other hand, appears to be mostly nuclear but during certain times of the cell cycle is clearly cytoplasmic (Fig. 4). Interestingly, Hel-N2 and HuA are the most similar to one another among all human members of the ELAV family in that they lack hinge segments and are expressed in proliferating cells (Gao et al., 1994; Ma et al., 1996; Gao and Keene, 1996; Antic and Keene, 1997). In this study, we report that HuA is located predominantly in the cytoplasm following mitosis and appears at that time to have an intracellular distribution very similar to the HuB proteins (Gao and Keene, 1996), before it later returns to the nucleus during G1. Nuclear-cytoplasmic movement of hnRNPA1 and other RRM proteins has also been demonstrated previously by blocking transcription with actinomycin D and DRB (Pinol-Roma and Dreyfuss, 1991, 1992). However, by direct comparison of HuA and hnRNPA1, we observed that HuA redistributed more extensively to the cytoplasm following these same treatments. It is logical to assume that HuA can interact with ERG mRNAs in the cytoplasm and as shown previously for HuB (Gao and Keene, 1996), would be available to affect mRNA stability and translation (Antic and Keene, 1997; Jain et al., 1997).

During revision of this manuscript, Fan and Steitz (1998) reported that the human homolog of HuA is a shuttling protein based upon its ability to move between nuclei in heterokaryons formed between human and mouse cells. Our data are consistent with HuA being able to shuttle between the nucleus and cytoplasm like other RNA binding proteins reported previously (Katz et al., 1994; Pinol-Roma and Dreyfuss, 1991, 1992; Zinszner et al., 1997; Peng et al., 1998). A well characterized type of nuclear export signal (NES) is a leucine-rich sequence first described in HIV-1 Rev (Fischer et al., 1994) and in PKI, a polypeptide inhibitor of the cAMP-dependent protein kinase (Wen et al., 1995). Bogerd et al. (1996) have shown the spacing between leucines (or isoleucines) is important and can be either 3-3-1 (Rex), 2-2-1 (Rev) or 3-2-1 (PKI) while the identity of these surrounding residues can vary. The sequence 78-I S T L N G L R L Q-87 in loop 5 of RRM 1 of mHuA is a potential NES of the Rev-type whose location may allow access to interacting factors. Interestingly, the U1-70K snRNP protein was found to contain a basic amino acid nuclear localization signal in the same position of the RRM structure (Romac et al., 1994). The differences in intracellular localization among ELAV family members cannot be solely controlled by this putative NES since this sequence exists in them all. The surrounding structure, including differences in potential phosphorylation sites which are unique to HuA, may control accessibility of the NES to interacting proteins. Phosphorylation is known to play an important role in the localization of many proteins (reviewed by Jans, 1995). Additionally, the intracellular distribution of ELAV members may result from a balance between their individual nuclear export and import.

**Upregulation of HuA expression and immune cell activation**

HuA levels increase dramatically following activation of mouse spleen T cells suggesting a possible role in immune function. T cell activation involving CD28 as the co-stimulatory molecule with CD3 has been shown to involve stabilization of cytokine mRNAs (Lindsten et al., 1989). Post-transcriptional cytokine mRNA stabilization mediated via the CD28 signaling pathway, allows activated T cells to escape apoptosis and secrete large amounts of cytokines (Noel et al., 1996). In contrast, cells activated solely via the TCR using only anti-CD3 secrete small amounts of cytokines before undergoing apoptosis. However, T cell stimulation does not result in indiscriminate ARE mRNA stabilization as seen by the stark increase in cytokine mRNAs in comparison with the small increase in protooncogene mRNAs following costimulation (Lindsten et al., 1989). This suggests that additional regulatory factors discriminate between these mRNAs. We report HuA upregulation during both mitogenic anti-CD3, as well as sub-mitogenic anti-CD3 and CD28 stimulation. Further experiments will be needed to understand the role(s) of HuA and other factors in regulating these two modes of T cell activation.

Umlauf et al. (1995) confirmed the importance of mRNA stabilization in T cell activation by detecting a 20-fold upregulation in IL-2 mRNA levels and up to a 100-fold increase in secreted IL-2 following T cell co-activation using both anti-CD3 and anti-CD28 but not only anti-CD3. Interestingly, nuclear pre-spliced IL-2 mRNA levels increased by 8-fold and they suggested that a transacting RNA binding protein capable of shuttling between the nucleus and cytoplasm may interact with IL-2 mRNA. In fact, three proteins which can bind to the AU-rich sequences of cytokine 3’UTRs as detected by UV-crosslinking were described by Bohjanen et al. (1991, 1992). Two of these, AU-B and AU-C, were upregulated during T cell activation. The third protein, AU-A, is a 34 kDa predominantly nuclear protein that is believed to shuttle between the nucleus and cytoplasm and is constitutively expressed. However, AU-A was reported not to be upregulated following T cell activation by the methods they used (Katz et al., 1994). Cloning of these factors has not been reported, nor have they been biochemically defined and it remains possible that AU-A and HuA are related proteins.

It is logical to expect that many proteins are upregulated during cellular stimulatory processes which result in
proliferation or metabolic activation. For example, other investigators have reported that some hnRNP proteins are elevated in PHA stimulated T cells (Biamonti et al., 1993). Since ELAV proteins have been demonstrated to stabilize ARE-containing mRNAs (Jain et al., 1997; Levy et al., 1998), the consequences of HuA upregulation for cytokine production in the immune system is an obvious possibility. Our results suggest that the ELAV protein, HuA, is an ideal candidate for a transacting factor which interacts with cytokine mRNAs and regulates their expression. These results do not rule out a role for HuA in also regulating proto-oncogene mRNA stabilities. Additional experiments will be necessary to determine whether these interactions directly influence cytokine production during immunoregulation.

**Implications for cell proliferation and differentiation**

Little is known about post-transcriptional regulation of the mammalian cell cycle. Protooncogenes, such as c-fos and c-myc are upregulated during the transition from $G_0$ to $G_1$, although the levels of c-foes are transient and c-myc remains high throughout the cell cycle. Among the known cyclins, only cyclins B1 and A are known to be regulated post-transcriptionally. In recent papers, Maita et al. (1995 and 1997) showed that the mRNA half-lives of both cyclins vary by as much as 4- to 5-fold during the cell cycle, although they peak at different times. The cyclin E mRNA half-life, however, did not appreciably change. Hence, the cell posttranscriptionally regulates cyclins B1 and A but not E. Although the 3'UTRs of both cyclin B1 and A contain multiple AU repeats (Hanley-Hyde et al., 1992; Ravnik and Wolgemuth, 1996), RNA binding proteins which interact with these mRNAs have not been elucidated. The overlapping temporal upregulation of HuA and cyclin B1 is intriguing.

We suggest that ERG products have evolved a default pathway of mRNA degradation as marked by the ARE in the 3'UTRs and that transacting factors like the ELAV proteins have the ability to reverse this effect by binding the mRNA instability sequence. The functions of ERGs in the seemingly distinct pathways of growth and differentiation may involve differences in localization by ELAV proteins and bound mRNA. Results with HuA reported here suggest that like Hel-N2, its expression correlates with increased proliferation during cell growth in culture, as well as in isolated spleen cells which are activated in vitro (Fig. 3). Although our data are consistent with a role for HuA in the cytoplasm, they do not preclude a role for HuA in RNA stability, splicing or mRNA transport, while in the nucleus (Antic and Keene, 1997, 1998). Data presented here are compatible with earlier hypotheses implicating mammalian ELAV proteins in the regulation of gene expression via their interactions with growth regulatory mRNAs (King et al., 1994; Gao and Keene, 1996; Antic and Keene, 1998). It is likely that they participate in both nuclear and cytoplasmic functions by binding to an mRNA subset and regulating their processing and/or transport during growth and development. It will be interesting to define other cellular components involved in these regulatory pathways via their interactions with ELAV proteins. These factors can be more readily investigated using a recently devised cell-free deacylation/degradation system in which HuA and HuB were shown to stabilize ARE-containing transcripts (unpublished data).

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**REFERENCES**


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