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LMO4 mRNA stability is regulated by extracellular ATP in F11 cells

Hsiao-Huei Chen [a,*], Jin Xu [a], Farzaneh Safarpour [a], Alexandre F.R. Stewart [b]

[a] Ottawa Health Research Institute, Neuroscience, Centre for Stroke Recovery, 451 Smyth Road, Ottawa, Ont., Canada K1H 8M5
[b] University of Ottawa Heart Institute, Ottawa, Ont., Canada

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Abstract

LIM only domain protein 4 (LMO4) interacts with many signaling and transcription factors to regulate cellular proliferation, differentiation and plasticity. In *Drosophila*, mutations in the 3′ untranslated region (UTR) of the homologue dLMO cause a gain of function by increasing mRNA stability. LMO4 3′UTR contains several AU-rich elements (ARE) and is highly conserved among vertebrates, suggesting that RNA destabilizing mechanisms are evolutionarily conserved. Here, we found that extracellular ATP stabilized LMO4 mRNA in F11 cells. The LMO4 3′UTR added to a luciferase reporter markedly reduced reporter activity under basal conditions, but increased activity with ATP treatment. Two ARE motifs were characterized in the LMO4 3′UTR. ATP increased binding of HuD protein to ARE1. ARE1 conferred ATP and HuD-dependent mRNA stabilization. In contrast, sequences flanking ARE2 bound CUGBP1 and ATP destabilized this complex. Thus, our results suggest that ATP modulates recruitment of RNA-binding proteins to the 3′UTR to stabilize LMO4 mRNA.

Keywords: LMO4; HuD; CUGBP1; ATP; RNA stability; AU-rich element

LMO4 is one of 4 LMO proteins in mammals that contain two zinc-finger LIM motifs for protein–protein interactions but lack a DNA binding domain. LMO proteins act as adaptors for the assembly of multiprotein complexes and regulate multiple pathways. For example, LMO4 promotes Stat3-dependent gene activation in response to IL-6 signaling [1] and acts as a co-activator in BMP/TGFβ signaling by interacting with receptor-activated smad proteins [2].

LMO4 is required for nervous system development [3]. LMO4 null mice die at birth with a defect in cranial neural tube closure and increased numbers of apoptotic cells in the neuroepithelium [4] suggesting LMO4 is required for neuron survival during developmental programmed cell death. In addition, LMO4 is required for proper cranial axon projections [5]. Conditional deletion of *lmo4* in postnatal cortical neurons causes aberrant segregation of thalamocortical afferents in layer IV and abnormal development of the somatosensory barrel cortex [6].

Deregulated expression of LMO4 is associated with breast [7], prostate [8] and oral cancers [9]. LMO4 forms a complex with CtIP and the tumor suppressor BRCA1 and inhibits BRCA1 transcriptional activity in breast epithelial cells [7]. In addition, LMO4 binds and represses estrogen receptor α leading to increased aggressiveness and invasiveness of breast cancer cells [10]. Moreover, LMO4 forms a complex with GATA6 and Ldb1 together with the LKB1 serine threonine kinase and activates the expression of the CDK inhibitor p21 that induces cell cycle arrest at G1/S [11]. Thus, through multiple interactions, LMO4 regulates cell proliferation and differentiation.

The 3′UTR of LMO4 is highly conserved between chicken, mouse and human over 300 million years of evolution (Fig. 1). It contains 3 adenine- and uridine-rich elements (ARE, 5′-AUUUA-3′) that control the expression of numerous genes by accelerating the decay of their mRNAs. In *Drosophila*, removal of the AU-rich elements (AREs) in the 3′UTR of dLMO increases the level of the dLMO transcript two- to fourfold higher than that of the wild type and results in a dominant wing scalloping phenotype [12].

* Corresponding author. Fax: +1 613 562 5403.
E-mail address: hchen@uottawa.ca (H.-H. Chen).

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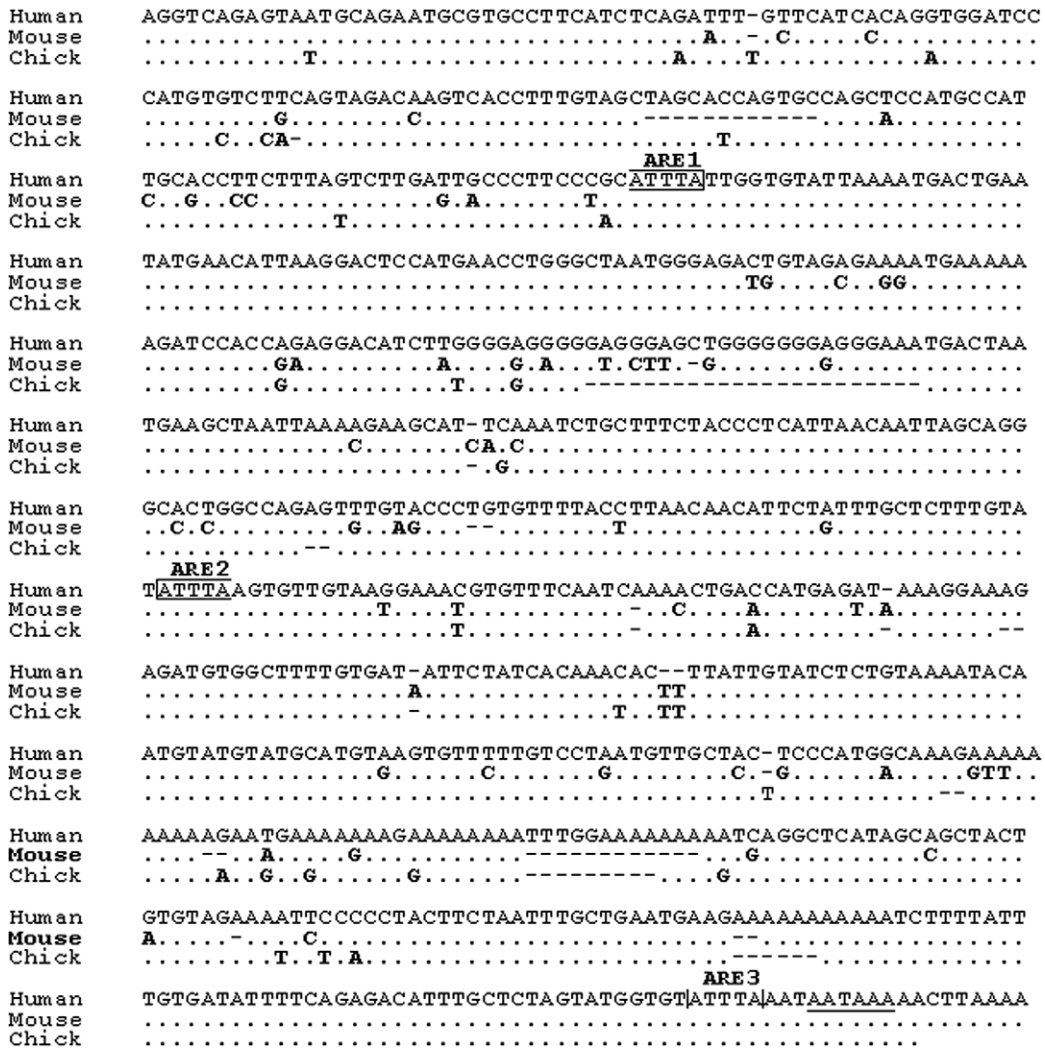

Fig. 1. Alignment of the human, mouse and chick LMO4 3′UTR reveals remarkable evolutionary conservation. Human and mouse sequences show 87% identity and human and chick are 93% identical. Sequences identical to human are shown by dots and dashes indicate gaps. ARE elements are boxed, and the polyadenylation signal is underlined.

The embryonic lethal abnormal vision (ELAV) family of RNA binding proteins, originally identified in *Drosophila* as essential for neural development [13], regulates RNA stability by binding to sequences containing AREs. Whereas HuR (or HuA) is a ubiquitously expressed ELAV protein, there are three neural-specific Hu family members in mammals: HuB, HuC, and HuD [14,15].

In renal mesangial cells, extracellular ATP promotes matrix metalloproteinase-9 (MMP9) expression by recruiting HuR to ARE motifs in the MMP9 3′UTR [16]. Extracellular ATP plays an important role in neuron cellular signaling [17], protects olfactory neurons by modulating sensitivity to toxins [18] and elevated extracellular ATP promotes neuron survival from the stress of starvation [19]. ATP activates purinergic receptors that elevate intracellular calcium [18]. Since LMO4 mediates activity-dependent transcription in cortical neurons in response to calcium influx [6], we investigated whether extracellular ATP could regulate LMO4 activity through an RNA stabilizing mechanism.

Here, we report that ATP upregulates LMO4 mRNA levels and that this effect is predominantly through increased mRNA stability. We have characterized regulatory mechanisms and show that ATP modulates the recruitment of RNA-binding proteins to the 3′UTR of LMO4 mRNA and this may account for regulated LMO4 expression in neurons.

Materials and methods

F11 cells. F11 cells, a fusion between rat E12 DRG neurons and a mouse neuroblastoma line were maintained as described [20]. Cells were treated with 100 μM ATP (Sigma) freshly prepared from the 10 mM stock (in water) for indicted time.

Northern blot analysis. Total RNA (10 μg) isolated from F11 cells with Trizol (Sigma) was analyzed by Northern blot using probes specific for LMO4 or glyceraldehyde phosphate dehydrogenase (GPDH) radiolabeled by random priming. 26S and 18S rRNA stained with ethidium bromide revealed the amount of RNA loaded. De novo RNA synthesis in F11 cells was blocked with actinomycin D (2.5 μg/ml) at the time of ATP treatment.

Plasmids and transfection. The 5′UTR of LMO4 was amplified using the following primer pairs: (F): 5′-ATTAAGCTTCTAGTTCTAGATC

GCGGGCGGCCCC-3′, (R): 5′-AACCATGGCCATGGTCTGCTTCT CCCCTATATTGCAAC-3′, digested with *Hin*dIII and *Nco*I and cloned upstream of the start site of luciferase cDNA in the pGL3P vector (Promega) (pGL3P5′UTR). The 3′UTR *Eco*RI/*Xho*I fragment of pSPORT6LMO4 (Open Biosystems, Image clone:3589532) was cloned downstream of luciferase cDNA between the *Xba*I and *Sal*I sites of pGL3P vector (pGL3P3′UTR or pGL3P5′3′UTR). ARE1 and ARE2 were mutated by site-directed mutagenesis (Stratagene) using the following primer pairs, ARE1(F): 5′-CTTGAGTACCCTTCCTGCAGTGATTG GTGTATTA-3′, ARE1(R): 5′-TAATACACCAATCACTGCAGGAAG GGTACTCAAG-3′, and ARE2(F): 5′-CTGTTTGCTCTTTGTATAGT GAAGTGTTGTAATGAAATGTG-3′, ARE2(R): 5′-CACATT TCATT ACAACACTTCACTATACAAAGAGCAAACAG-3′. HuD expression vector was described previously [21].

Western blot analysis. F11 cells were harvested in RIPA buffer with proteinase inhibitors (Sigma) and 40 μg of protein extract were analyzed as described previously [22].

Antibodies. Antibodies to HuD (16C12, Clongene), pan-Hu and CUGBP1 (SantaCruz, Inc.) and actin (Sigma) were used for gel supershift or Western blot analysis. LMO4 peptide-specific antibody was custom ordered (Zymed).

RNA gel shift. RNA gel shifts were carried out as described [23]. RNA oligonucleotides (IDT Technologies) were: ARE1, 5′-UUCCUG CAUUUAUUGGUGUAU-3′, mARE1, 5′-UUCCUGCAGUGAUU GGUGUAU-3′, ARE2, 5′-UUUGUAUAUUUAAGUGUUGUA-3′, and mARE2, 5′-UUUGUAUAGUGAAGUGUUGUA-3′. Cytoplasmic proteins (6 μg each) were prepared as described [16] and incubated with the RNA probes under RNase-free conditions and analyzed on non-

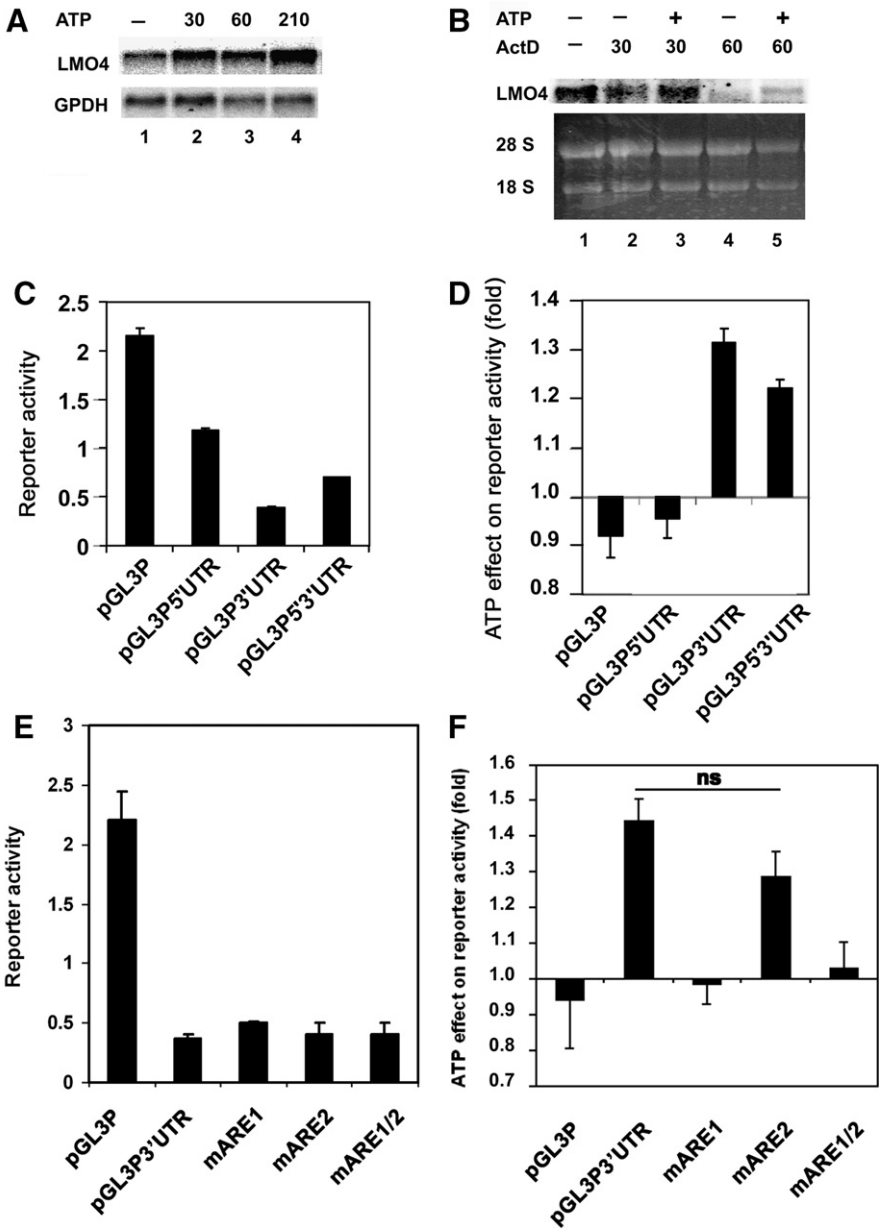

Fig. 2. Extracellular ATP upregulates and stabilizes LMO4 mRNA in F11 cells. Northern blot showed increased LMO4 mRNA levels 30, 60, or 210 min after ATP treatment (A), even in the presence of actinomycin D (B). (C) Both the 5′ and 3′UTR sequences of LMO4 destabilized a pGL3P-luciferase reporter ($n = 9$). (D) ATP treatment inhibited control pGL3P and pGL3P5′UTR reporter activity but increased the reporter activity bearing the 3′UTR ($n = 6$). (E) Mutation of neither ARE1 nor ARE2 increased stability of the LMO4 3′UTR under basal conditions ($n = 9$). (F) Mutation of ARE1 ($p < 0.01$), but not ARE2, was sufficient to block ATP-dependent stabilization of LMO4 mRNA. ns, not significant ($n = 6$). (D) and (F) are expressed as fold relative to basal conditions.

denaturing 6% polyacrylamide gels. To identify the proteins bound to the RNA probes, 1 μg of antibodies were added. For competition experiments, a 50-fold molar excess of unlabeled RNA oligonucleotide was used.

UV cross-linking. Binding of cytoplasmic extracts to radiolabelled RNA oligonucleotides was carried out as above, and reactions were exposed to 120 mJ of UV using a Stratalinker (Stratagene). RNA/protein complexes were analyzed on 12% SDS–polyacrylamide gels and revealed by autoradiography using a phosphor storage screen (GE).

Results and discussion

The 3′UTR of LMO4 (Fig. 1) is more highly conserved than the GAP43 mRNA that shows 70% identity over 1400 nucleotides [24]. Like *Drosophila* dLMO, mammalian LMO4 mRNA is also regulated by ARE sequences in the 3′UTR, suggesting an important conserved function of the LMO4 3′UTR.

Extracellular ATP increases LMO4 mRNA levels in the dorsal root ganglia-derived F11 cells (Fig. 2A). This effect is not dependent on transcription, since treating cultured neurons with actinomycin D did not prevent the ATP mediated increase. LMO4 mRNA is highly labile under basal conditions, with a half-life of less than 15 min (Fig. 2B). These results suggested that LMO4 mRNA is stabilized by extracellular ATP. To test whether non-coding sequences of the LMO4 mRNA regulate its stability, constructs bearing the 5′UTR and/or the 3′UTR flanking a luciferase reporter were made. Both the 5′ and 3′UTR sequences of LMO4 decreased the activity of the pGL3P-luciferase reporter (Fig. 2C). ATP treatment inhibits control pGL3P and pGL3P 5′UTR reporter activity, whereas the reporter bearing the 3′UTR showed increased reporter activity 3.5 h after ATP treatment (Fig. 2D). In contrast to the 3′UTR, the 5′UTR did not increase mRNA stability in response to ATP. This suggests that all of the functional response to ATP resides in the 3′UTR of LMO4.

Three AREs were identified in the LMO4 3′UTR (Fig. 1). Mutation of either the ARE1 or the ARE2 motif within the context of the pGL3P3′UTR reporter plasmid had little effect on the basal activity of this reporter vector, suggesting that neither sequence individually nor together accounts for RNA destabilization (Fig. 2E). However, the ATP response of pGL3P3′UTR was abrogated by the ARE1 mutation, whereas the 3′UTR carrying a mutation of ARE2 retained its response to ATP (Fig. 2F). These results indicate that ARE1 is necessary and sufficient to convey the ATP response.

To identify the ARE1 binding factors, gel electrophoretic mobility shift assays were carried out using a synthetic RNA oligonucleotide and cytoplasmic extracts from F11 cells isolated under control conditions or following treatment with ATP for 3.5 h. Using an RNA oligonucleotide containing the wild-type ARE1 motif from the 3′UTR of LMO4, we observed 2 shifted complexes (Fig. 3A). Interestingly, binding of these complexes was increased when cells were treated with ATP (lane 3). An unlabelled mutant ARE1 competed for binding to the lower mobility complex (ns), but less so with the

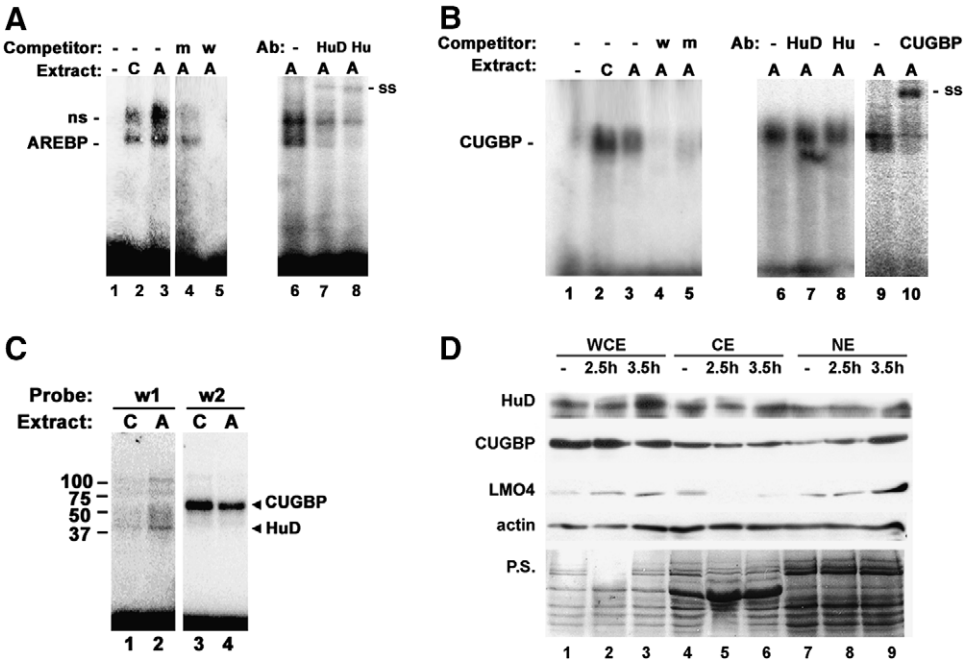

Fig. 3. ATP recruits HuD to the ARE1 element of LMO4 mRNA 3′UTR. Gel mobility shift assays using ARE1 (A) or ARE2 (B) RNA oligonucleotides with control or ATP treated F11 extract. Wild type (w) or mutant (m) ARE1 or ARE2 unlabelled oligonucleotides were used as competitors. HuD, pan-Hu or CUGBP1 antibodies were used to supershift (ss) RNA-binding complexes. Results are representative of 4 experiments. (C) UV cross-linking of cytoplasmic extracts to the ARE1 and ARE2 RNA oligonulceotides were size-fractionated by SDS–PAGE. (D) Whole cell (WCE), cytoplasmic (CE) and nuclear extracts (NE) from control (−) and ATP-treated F11 cells (for 2.5 or 3.5 h) were analyzed by Western blot. Ponceau staining (PS) shows total proteins on the blot. Results are representative of 3 separate experiments.

higher mobility complex (AREBP, lane 4). In contrast, unlabelled ARE1 competed for binding of both complexes (lane 5). Gel supershift assays using HuD and pan-Hu antibodies demonstrated that HuD binds to ARE1 (lanes 9 and 10).

Unlike ARE1, wild-type ARE2 produced a single shifted complex and ATP treatment reduced binding (Fig. 3B). Both wild-type and mutant ARE2 unlabeled oligonucleotides competed for binding to ARE2 (lanes 3 and 4). Thus, these results suggested that the shifted complex does not bind to the 5′-AUUUA-3′ motif at the core of ARE2, but rather to flanking sequences within the oligonucleotide. Examination of the ARE2 oligonucleotide revealed three 5′-UGU-3′ motifs on either side of the ARE2 that could serve as binding sites for CUGBP1. Consistent with this idea, the HuD or pan-Hu antibodies did not supershift (lanes 7 and 8), whereas CUGBP1 antibody supershifted the ARE2-bound complex (lane 10). Moreover, our data suggest that CUGBP1 binding to the ARE2 oligonucleotide is inhibited by ATP (compare lanes 2 and 3). Marquis et al. showed a minimal requirement of three tandem UGU motifs for strong CUGBP1 binding [25]. Similarly, we found CUGBP1 binding only to the ARE2 oligonucleotide containing 3 UGU motifs, but not to ARE1 or ARE3 (data not shown), each containing only one UGU.

UV cross-linking confirmed that ARE1 and ARE2 bind to distinct protein complexes (Fig. 3C). ARE1 showed a predominant protein at 40 kDa (consistent with HuD), whereas ARE2 bound to a protein of approximately 56 kDa (consistent with CUGBP1). ATP treatment increased the amount of protein cross-linked to ARE1 but decreased ARE2 cross-linked protein, consistent with the gel shift results. CUGBP binds to the c-fos and TNFα mRNA and stimulates poly(A) shortening by recruiting a deadenylase, the polyadenyl ribonuclease PARN, to these RNA substrates [26]. Consistent with a role for CUGBP1 in RNA degradation, reduced binding of CUGBP1 is associated with increased LMO4 mRNA levels. Ours is the first report to show that extracellular ATP reduces CUGBP1 binding to a labile mRNA.

Western blot analysis showed that HuD was present in both the cytoplasm and nucleus and this distribution was not changed by ATP treatment (Fig. 3D). However, ATP induced translocation of CUGBP1 to the nucleus. This may account in part for reduced binding to the ARE2 oligonucleotide in gel mobility shift assays, increased LMO4 mRNA stability and increased activity of the pGL3P3′UTR reporter. Stabilization of LMO4 mRNA was associated with increased LMO4 protein levels (Fig. 3D) after ATP treatment.

To test whether HuD binding to ARE1 stabilizes the LMO4 3′UTR, HuD was over-expressed together with the pGL3P3′UTR reporter plasmids. HuD increased the activity of the wild type reporter under basal conditions (Fig. 4A). A similar effect of HuD over-expression in cortical neurons was observed on the stabilization of the GAP43 mRNA [21]. Moreover, ATP treatment augmented the response to HuD over-expression and mutation of ARE1 completely abolished this response (Fig. 4B). Thus, in addition to reducing CUGBP1 binding to ARE2, ATP promotes HuD binding to ARE1 to stabilize the LMO4 3′UTR and permit high levels of reporter activity. In addition, LMO4 levels were more prevalent in the nuclear fraction (Fig. 3D) with ATP treatment. Taken together, our study suggests that ATP-induced stabilization of LMO4 mRNA and nuclear translocation of LMO4 protein can contribute to changes in gene expression in neurons.

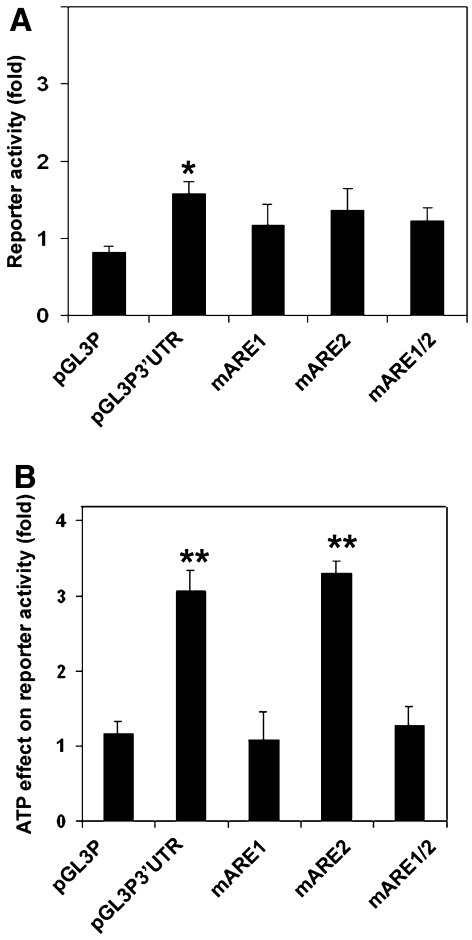

Fig. 4. Over-expression of HuD augments the activity of a reporter bearing the LMO4 3′UTR (A) under basal conditions ($^{*}p > 0.05$) and in response to ATP (B). The ATP response to HuD was abolished by mutation of ARE1 ($^{**}p < 0.01$, $n = 4$). Data are expressed as fold relative to the control empty expression vector.

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