Lactate Dehydrogenase Is an AU-rich Element-binding Protein That Directly Interacts with AUF1*

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The regulation of mRNA turnover is a key mechanism of modulating eukaryotic gene expression. Labile cytokine, lymphokine, and proto-oncogene messages contain AU-rich sequences in their 3′-untranslated regions that are highly conserved across mammalian species. As demonstrated conclusively by Shaw and Kamen (1), these AU-rich elements (ARE) compose a major class of cis-acting stability determinants. These ARE consist of reiterations of the pentamer AUUUA or oligo(U) sequences in an AU-rich context (1–3). Significantly, cytoplasmic and nuclear proteins have been identified that bind specifically to the ARE; these AU-binding proteins (AUBP) are thought to regulate mRNA stability in trans (reviewed in Ref. 4). Although a variety of these AUBP has been described, functional roles in ARE-dependent turnover have been attributed to a comparatively small number of proteins, including HuR and AUF1 (5–12).

HuR, a ubiquitously expressed member of the Elav family of RNA-binding proteins, interacts in vitro with the ARE of c-fos and IL-3 mRNA (5), as well as with synthetic mRNA-destabilizing ARE (6). Several studies (7, 8) have demonstrated that overexpression of HuR results in stabilization of reporter transcripts containing the ARE of GM-CSF, c-fos, and vascular endothelial growth factor. These studies did not demonstrate altered protein production as a consequence of increased mRNA accumulation (5–8). Similarly, in vitro ARE binding activity has been demonstrated for AUF; this protein was initially purified because of its ability to accelerate the degradation of c-myc mRNA in an in vitro decay system (9, 10). However, recent work (11, 12) has suggested AUF1 may play multiple roles in the regulation of mRNA turnover. For example, Kiledjian et al. (11) have demonstrated that AUF1 is a component of the α-globin mRNA stabilization complex, whereas other studies (12) have implicated AUF1 in B-cell transcriptional activation. The diversity of functions attributed to AUF1 suggests its activity may be regulated by protein-protein interactions.

Our laboratory has been examining potential trans-acting factors involved in mediating ARE-dependent mRNA turnover. Initial studies centered on investigating the role of hnRNP A1, prompted by its discovery as the major cytoplasmic protein capable of binding the GM-CSF ARE in activated T lymphocytes (13, 14). In addition, IL-2 overproduction by a retrovirally infected T cell line, MLA-144, was shown to be due to increased mRNA stability (15). Stabilization of IL-2 mRNA correlated with a proviral insertion in its 3′-UTR which enhances the binding of hnRNP A1 to its ARE relative to that of native IL-2 (15).

To examine the role of hnRNP A1 in ARE-dependent mRNA turnover, we utilized the DP28-9, CB7, and CB3 murine erythroleukemia (MEL) cell lines, which vary in their expression of ARE-dependent electrophoresis; hnRNP, heterogeneous nuclear ribonucleoprotein; Pipes, 1,4-piperazinediethanesulfonic acid; MEL, murine erythroleukemia; IL, interleukin; CAPS, 3-(cyclohexylamino)propanesulfonic acid.
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Friend leukemia virus (FV-P) and contain two functional hnRNP A1 genes (16). In contrast, the CB7 MEL cell line has only one active hnRNP A1 gene, due to silencing of one allele by a downstream insertion of F-murine leukemia virus helper virus at the Fli-2 locus. In CB3 MEL cells, proviral integration occurred at both Fli-2 loci, resulting in the absence of hnRNP A1 mRNA and protein (16). Transient as well as stable transfection experiments of CB3 MEL cells with hnRNP A1 failed to indicate an effect of hnRNP A1 on ARE-dependent mRNA turnover or expression of reporter gene constructs. Similarly, overexpression of hnRNP A1 in human Jurkat T cells did not result in alteration of ARE-dependent gene expression.

As we were unable to detect an effect of hnRNP A1 on ARE-dependent gene regulation, we examined the AUBP profiles of the three cell lines. The major cytosolic protein capable of binding the ARE of GM-CSF in the context of its full-length RNA in CB3 erythroleukemia cells was identified as l-lactate dehydrogenase (LDH) isozyme M. Lactate dehydrogenase, which catalyzes the reversible conversion of pyruvate to lactate, is a tetrameric enzyme that may exist in several enzymatic forms (17). Each LDH subunit is one of two types, designated H (for heart) and M (for skeletal muscle). The five tetrameric isozymes of LDH are five different combinations of these subunits (17). The isozyme of LDH identified in these studies is LDH-M, which consists of five M polypeptide subunits. (For purposes of ease of reference, the LDH-M will be referred to as simply LDH throughout the text.). LDH was shown to bind specifically to the ARE of GM-CSF RNA using both UV cross-linking and filter binding assays. LDH is polysomally associated and coimmunoprecipitates with AUF1 and hsp-70. These finding suggest that LDH may serve multiple roles in RNA metabolism beyond its role in glycolysis. Indeed, non-overlapping roles of LDH are suggested by the fact that the binding of the ARE by LDH is competed by NAD+, indicating that each utilizes the Rossmann fold. Similar roles have been well defined for the iron-response element-binding protein and its dual role as an aconitase (18, 19). Collectively, these data implicate LDH as a functionally relevant AUBP and prompt consideration of an expanded role of this enzyme in the post-transcriptional regulation of gene expression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**Mouse erythroleukemia (MEL) cell lines (DP28-8, CB3, and CD7) were generously provided by Benoit Chabot and Yuriy Ben-David. MEL, Jurkat, and THP-1 monocytic cell lines were maintained in RPMI 1640 medium (Cellgro) supplemented with 10% heat-inactivated newborn calf serum (HyClone) and 50 μg/ml gentamicin sulfate (Sigma) at a density of 5 × 10⁶ cells/ml.

CB3C7-11 and CB3C7-20 MEL cells (stably transfected with antisense and sense hnRNP A1, respectively, generously provided by Benoit Chabot) were transiently transfected with 0.1 μg of pG33 luciferase reporter DNA (Promega) containing six reiterations of either AUAUA or AUGUA in their 3′-UTR by LipofectAMINE (Invitrogen). Jurkat cells were cotransfected with either of the AUAUA or AUGUA pG33 constructs as well as pCMV-A1 vector (gift of Benoit Chabot). CB3 MEL cells were stably transfected with human GM-CSF cDNA (generously provided by James Malter). Transient transfections were incubated in serum-free RPMI 1640 for 24 h; at the conclusion of this incubation, RMPI-1640 medium supplemented with 15% fetal calf serum (HyClone) was added to the transfections. After 20 h, cells were analyzed for luciferase expression using luciferase assay kit (Promega).

**Preparation of in Vitro Transcripts—**RNA transcripts were generated from linearized DNA templates in the presence of 50 μCi of [α-32P]UTP (Perkin-Elmer Life Sciences), 20 μM UTP, and 4 mM each ATP, GTP, and CTP (Amersham Biosciences) and phenol/chloroform was extracted, ethanol-precipitated, and applied to P30 BioSpin Columns (Bio-Rad). The XhoI fragment of the pXM vector containing human GM-CSF DNA (provided by Genetics Institute) was subcloned into the multiple cloning site of the pT7/73x19 plasmid at its BamHI site. The wild type GM-CSF RNA probe was synthesized by T7 polymerase transcription of this plasmid linearized with EcoRI. The mutated GM-CSF RNA probe (containing AUGUA reiterations in lieu of AUUUA pentamers) was generated by PCR and confirmed by sequencing.

**RNA Binding Analysis by UV Cross-linking—**RNA probes (8 × 10⁵ cpm) were incubated with either 25 μg of purified cytoplasmic lysate or 1 μg of commercially prepared rabbit muscle lactate dehydrogenase (Roche Molecular Biochemicals) in 12 mM Hepes, pH 7.9, 15 mM KCl, 0.2 μM dithiothreitol, 0.2 μM yeast tRNA, and 10% glycerol (all purchased form Sigma) for 10 min at 30 °C and then UV cross-linked on ice for 5 min with 1 M uracil-β-d-thyminederivative (Bio-Rad). Reaction mixtures were digested with 15 μg of RNase A and 7.5 units of RNase T1 (Roche Molecular Biochemicals) for 30 min at 37 °C, solubilized in Laemmli SDS sample buffer (21), and analyzed by 15% SDS-PAGE and autoradiography. Two-dimensional NEPHGE was performed with separation in the first dimension accomplished utilizing a pH 3–10 ampholyte gradient (Bio-Rad) for 2 h and 15 min at 400 V (22). Separation in the second dimension was achieved by 15% SDS-PAGE.

**Protein Purification—**For p36 AUBP purification, a 20-liter culture of CB3 MEL cells was harvested. Cells were washed in ice-cold 1X PBS, and cell pellets were resuspended in buffer A, containing 10 mM Pipes, pH 6.8, 100 mM KCl, 2.5 mM MgCl₂, 300 mM sucrose, 1 mM Pefabloc (Invitrogen), and then lysed by the addition of Triton X-100 (1% final) (Sigma) on ice for 5 min (23). Supernatants were collected, aliquoted, and stored at −80 °C. Nuclei were pelleted, and cytoplasmic lysate was sequentially precipitated with the addition of 25, 35, 45, 50, and 75% ammonium sulfate and then dialyzed against 0.5X PBS, 1 mM Pefabloc, 5% glycerol. The 50% ammonium sulfate fraction (which contained the 36-kDa AUBP activity) was resuspended in buffer B (50 mM Hepes, pH 7.9, 25 mM KCl, 5 mM MgCl₂, 0.5 mM dithiothreitol, 1 mM Pefabloc), and subjected to carboxymethylcellulose column chromatography with stepwise salt elution. The 0.25 M KCl eluent was desalted with a P6 BioSpin Column (Bio-Rad), resuspended in 0.1 M KCl, and applied to a poly(U)-Sepharose (Amersham Biosciences) column. The 36-kDa AUBP activity was eluted from poly(U)-Sepharose in the 1 M KCl fraction. This chromatographic fraction was aliquoted and stored at −80 °C. Protein concentrations were determined by BCA protein assay (Pierce). The RNA binding assay delineated above was used to assess the RNA binding activity of each fraction.

**Immunoprecipitation—**UV cross-linking reactions contained CB3 polypeptides (10 A₈₀⁰) to GM-CSF RNA. Reactions were digested with RNase and then immunoprecipitated with a rabbit polyclonal antibody to rat heart LDH (AB1222) (Chemicon). Immunoprecipitates were captured with protein G-agarose beads (Bio-Rad) overnight at 4 °C, and beads were washed six times in 100 mM NaCl. Proteins were resolved by 15% SDS-PAGE and electrotransferred to nitrocellulose membrane in CAPS (Sigma) buffer, pH 11.0, with 15% methanol. Immunoblots were washed with Tris-buffered saline, 0.1% Tween 20 (Sigma) and blocked in 5% milk overnight at 4 °C. Membranes were then probed with Anti-rabbit IgG goat anti-α (Pierce) and peroxidase-labeled horseradish peroxidase (Bio-Rad). Common precipitation of LDH with AUF1 was detected by immunoprecipitating with either THP-1 polypeptides or CB3 polypeptides (10 A₈₀⁰) with α-AUF1 polyclonal antibody, followed by immunoblotting with AB1222. The reciprocal experiments were conducted utilizing the same antibodies. The interaction between LDH and hsp-70 was detected by immunoprecipitating with AB1222 and then immunoblotting with α-hsp-70 monoclonal antibody (W27) (Santa Cruz Biotechnology). Depleted lysates represent supernatants of immunoprecipitations. Reactive antigens were visualized with Supersignal chemiluminescence substrate (Pierce).

**Nitrocellulose Filter Binding Assay—**Purified LDH and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Roche Molecular Biochemicals) were incubated with 50,000 cpm radiolabeled GM-CSF RNA in binding buffer (10 mM Hepes/KOH, pH 7.4, 100 mM KCl, 2.5 mM MgCl₂, 50 mM/mg competitor yeast tRNA) in a final volume of 20 μl for 30 min at 30 °C as described previously (23). For binding competition experiments, NAD⁺ (Sigma) was preincubated with reactions for 5 min at room temperature. Reactions were then applied to nitrocellulose under negative pressure and briefly washed with binding buffer. Filters were air-dried and used for liquid scintillation. Binding curves from five different experiments (each performed in triplicate) were used to determine the dissociation constant (Kₐ) (24).

**Polyosome Profile Analysis by Sucrose Density Gradient—**4 × 10⁶ CB3 MEL or THP-1 monocytic cells were resuspended in buffer C (10 mM Tris-Cl, pH 7.5, 1 mM potassium acetate, 1.5 mM magnesium acetate, 2 mM dithiothreitol, 1.5 μl per 10⁶ cells) (25). MEL and THP-1 cells
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RESULTS

Characterization of ARE-dependent mRNA Turnover in an hnRNP A1-deficient Cell Line—Previous studies (13, 14) identified hnRNP A1 as the dominant cytoplasmic protein capable of specifically interacting with the ARE of full-length GM-CSF mRNA in activated T lymphocytes. To examine the role of hnRNP A1 in mRNA turnover, we utilized MEL cell lines in which hnRNP A1 expression had been silenced by retroviral insertion (16). Insertion of the Friend-murine leukemia virus helper virus at the Fli-2 locus silenced expression from either one (CB7) or both (CB3) hnRNP A1 alleles. Immunoblotting established the reported absence of hnRNP A1 in the CB3 cell line was not due to altered subcellular distribution (Fig. 2A). In contrast, the CB3 cell line overexpressed hnRNP A2, particularly in the cytoplasmic and polysomal fractions (Fig. 2A), perhaps to compensate for the absence of hnRNP A1.

The role of hnRNP A1 in ARE-dependent mRNA turnover was analyzed. CB3 cells stably transfected with hnRNP A1 or a control cDNA were analyzed by transient transfection for different expression of luciferase reporter genes into which 6 consecutive repetitions of AUUUA or AUGUA had been engineered into their 3'–UTR. The presence of reiterated AUUUA pentamers reduced luciferase expression by 70–90% relative to controls or vectors with AUGUA sequences, indicating the posttranscriptional activity of this ARE. There was no difference in ARE-dependent inhibition of luciferase expression in CB3 cells that lacked or contained hnRNP A1 (data not shown). Similarly, ARE-mediated inhibition did not differ between the DP28-9, CB7, and CB3 cell lines (data not shown). Moreover, no consistent effect on GM-CSF mRNA turnover was observed with CB3 cells (that differed only in hnRNP A1 expression) stably transfected with the human GM-CSF cDNA (data not shown). Finally, overexpression of hnRNP A1 in the human Jurkat T cell line had no effect on ARE-dependent luciferase expression (data not shown). Based on these studies, we concluded that in these cell lines and under these conditions, hnRNP A1 plays either a redundant or minor role in the posttranscriptional regulation of GM-CSF and ARE-dependent mRNA turnover.

Characterization of AUBP Proteins in an hnRNP A1-deficient Cell Line—Given the lack of effect of hnRNP A1 on ARE-dependent gene expression, the presence of other AUBP which mediated ARE-dependent mRNA turnover was examined by concurrent UV cross-linking and immunoblotting. In these experiments, in vitro transcribed, 32P-labeled full-length GM-CSF RNA was incubated with cytoplasmic lysate from DP28-9, CB7, and CB3 cells, UV cross-linked, RNase-digested, resolved by SDS-PAGE, and blotted onto nitrocellulose. As described previously (13) in T lymphocyte cytosols, a single 36-kDa protein capable of binding the ARE of GM-CSF mRNA in the context of full-length RNA was observed, which colocalized with hnRNP A1. In DP28-9 and CB7 cytosols, (Fig. 1, B and C), a 36-kDa GM-CSF RNA binding activity paralleled hnRNP A1 levels, whereas the identity of the 36-kDa-binding protein (hereafter referred to as p36) in the CB3 cells was unknown, as no hnRNP A1 was present. The p36 binding activity was ARESpecific, as no RNA binding activity was seen with mutant GM-CSF RNA probe (in which the central uridine of the canonical ARE had been changed to guanosine) (Fig. 1B). Immunoprecipitation approaches and two-dimensional NPHGE immunoblots of the UV cross-linked binding activity indicated that the p36 binding activity was not hnRNP A2, a member of the Elav family (HuR and Hel-N1) of RNA-binding proteins, GAPDH or AUF1 (see Ref. 26 and data not shown). We therefore concluded that this protein represented a novel AUBP.

Identification of the p36 AU-specific RNA-binding Protein as Lactate Dehydrogenase—The p36 AUBP was initially purified by ammonium sulfate precipitation of CB3 cytoplasmic lysate (Fig. 2A). The majority of 36-kDa AUBP activity eluted in the 50% ammonium sulfate fraction (Fig. 2B). Carboxymethylcel lulose chromatography resulted in the elution of this RNA binding activity at 0.25 M KCl (Fig. 2B). This 0.25 M fraction was further purified by poly(U)-Sepharose chromatography, with the 1 M KCl elution containing the RNA binding activity (Fig. 2B). Immunoblotting the 1 M poly(U)-Sepharose elution
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**FIG. 2.** Isolation of p36 AUBP and identification as LDH-M. A, purification scheme. B, AUBP analysis of each purification stage, as described in Fig. 1B. C, CB3 polysomes (0.5 A260) and 1 mM poly(U)-purified eluate (50 μg) were UV cross-linked to [32P]UTP-labeled wild type GM-CSF RNA, RNase-digested, and then analyzed by two-dimensional NEPHGE and autoradiography. D, CB3 cytoplasmic lysate (200 μg) was UV cross-linked to [32P]UTP-labeled wild type GM-CSF RNA, RNase-digested, and immunoprecipitated with polyclonal anti-LDH antibody (AB1222). Beads were washed and then analyzed by 15% SDS-PAGE and autoradiography. Lanes labeled 2, 4, and 6 represent second, fourth and sixth washes, respectively, indicating elimination of nonspecific binding activity.

![Diagram of purification scheme](image)

Demonstrated no HuR or AUF1 (data not shown). Two-dimensional NEPHGE analysis of AUBP activity in the 1 mM poly(U) fraction revealed the presence of three isoforms of a 36-kDa protein, with pI values between 6.6 and 8.0 (Fig. 2C). This pattern was identical to that found using CB3 polysomes, indicating purification of the targeted binding activity. The 36-kDa protein in the 1 mM poly(U) fraction resolved as a single band on 15% SDS-PAGE that was excised, digested in situ with trypsin, and analyzed by matrix-assisted laser desorption/ionization-mass spectrometry. This analysis identified the 36-kDa AUBP as murine L-lactate dehydrogenase M with a probability score of 9.9 × 10^{-11}. To verify that the p36 RNA binding activity in the CB3 cytosol was LDH, a rabbit polyclonal anti-LDH antibody was used to immunoprecipitate the radiolabeled RNA-protein complex from UV cross-linked CB3 cytoplasmic lysates (Fig. 2D).

**FIG. 3.** LDH specifically binds GM-CSF RNA AURE in nitrocellulose filter binding assay. Shown is the ability of purified LDH to bind specifically to the ARE of GM-CSF RNA, as determined by nitrocellulose filter binding assay. Indicated amounts of purified LDH protein were incubated with [32P]UTP-labeled (80,000 cpm) wild type or mutant GM-CSF RNA, or globin pre-mRNA and analyzed for binding to nitrocellulose by scintillation counting. Data are shown as the % bound of input RNA.

**LDH Binding Specificity for GM-CSF mRNA in a Non-UV Cross-linking Assay**—Nitrocellulose filter binding assays examined the ability of LDH to bind specifically to the ARE of GM-CSF RNA independent of cross-linking. LDH/GM-CSF RNA complex formation plateaued at 750 nM with ~75% of input RNA bound (Fig. 3). Binding of the mutant AUGUA GM-CSF RNA probe was markedly diminished, approximating that seen with a radiolabeled globin pre-mRNA transcript, which lacks an ARE. Based on these studies, the K_d of purified LDH binding to the wild type GM-CSF RNA was 501.9 nM. In contrast, the K_d of the LDH binding to the mutated GM-CSF RNA was determined as 1879 nM, confirming its ARE specificity.

Studies have demonstrated the RNA binding capabilities of several glycolytic enzymes (reviewed in Ref. 27). Each of these enzymes utilize NAD^+ and bind to AREs (reviewed in Ref. 28). Intriguingly, proteolysis studies have implicated the Rossmann fold as the RNA binding domain of GAPDH (20). The identification of this putative RNA binding domain was supported by the ability of NAD^+ to interfere with RNA binding (20). Incubation of purified LDH with NAD^+ prior to UV cross-linking inhibited AUBP activity in a concentration-dependent manner and was eliminated at 10 μM NAD^+ (Fig. 4A). This effect was independent of UV cross-linking, as similar results were seen in filter binding assays (Fig. 4B). Collectively, these data show that occupancy of the Rossmann fold by NAD^+ inhibits RNA binding by LDH, suggesting that LDH utilizes the Rossmann fold for binding either NAD^+ or the ARE of GM-CSF RNA.

**Polysomal Localization of LDH Is Dependent on RNA Binding**—With the identification of LDH as a cytoplasmic GM-CSF RNA-binding protein, its subcellular localization was examined. Immunoblotting demonstrated that a discrete percentage of LDH is polysomal (Fig. 5A). Comparison of various cell equivalents of S130 to a fixed amount of CB3 polysomes indicated that polysomal levels of LDH are ~10% those of cytoplasmic LDH in CB3 cells (data not shown). Discontinuous (10–40%) sucrose density gradient analysis demonstrated that similar ratios of polysomal LDH were detected in cells containing hnrNP A1, including THP-1 monocytic cells and activated human T lymphocytes (Fig. 5B and data not shown). LDH was predominantly found in the 40% sucrose density fraction, suggesting association with mRNA that are being actively translated. Disruption of mRNP complexes from ribosomes by EDTA...
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FIG. 4. NAD inhibits RNA binding by LDH. A, inhibition of AUBP activity by UV cross-linking assay. Purified LDH protein (0.5 μg) was preincubated for 5 min at room temperature with the indicated concentrations of NAD prior to binding and UV cross-linking to [32P]UTP-labeled wild type GM-CSF RNA. B, purified LDH protein (0.5 μg) was preincubated with indicated concentrations of NAD for 5 min at room temperature and then analyzed for binding to radiolabeled wild type GM-CSF RNA by nitrocellulose filter binding and scintillation counting.

Polysomal LDH Interacts with AUF1—The dependence of the polysomal localization of LDH on RNA binding suggests that LDH interacts directly with RNA in vivo. In the absence of RNA binding, some LDH remained in the 20% sucrose density fraction (refer to Fig. 5B), suggesting it is part of a larger complex of proteins. In addition, the polysomal distribution of AUF1 to the most dense polysomal fraction (fraction number 18, as indicated by sucrose density continuous gradient analysis) was similar to that seen with LDH in MEL cells as well as THP-1 monocyctic cells, suggesting their possible interaction (Fig. 6). Interestingly, there appears to be a greater distribution of LDH to the dense polysomal fractions of CB3 versus THP-1 cells. This may reflect differences in the cellular distribution of LDH between erythroid (CB3) and myeloid (THP-1) cells and/or species (mouse versus human). Alternatively, the absence of hnRNP A1 in the CB3 cell line may play a factor and would suggest that LDH serves some other compensatory function or competes with hnRNP A1 for mRNA-binding sites in the cytoplasm. This association was confirmed by finding that polysomal LDH and AUF1 could be coimmunoprecipitated (Fig. 7A). Based on densitometry, 50% or greater of polysomal LDH is bound to AUF1 in the CB3 and THP-1 cell lines. Immunoprecipitation of LDH from THP-1 polysomes demonstrated the presence of AUF1 and hsp-70, which were shown previously to interact in HeLa cytosol (31).

In order to distinguish if the interaction between LDH and AUF1 was direct, and not due to coassociation of an mRNA, in vitro binding studies were undertaken in the absence of RNA. His-tagged recombinant p40 AUF1 was bound to metal affinity beads and incubated with purified LDH. Following washing, bead-bound complexes were eluted, resolved by SDS-PAGE, and visualized. As demonstrated in Fig. 7B, ~73% of the input LDH (determined densitometrically) bound the p40 isoform of AUF1. Thus LDH and AUF1 interact directly in the absence of RNA and other proteins. Given the immunoprecipitation results, the data demonstrate the interaction of LDH, AUF1, and hsp-70 occur in the polysomal compartment and suggest it is mediated through direct protein-protein interaction.

DISCUSSION

Although identified as a major AUBP in human and gibbon T lymphocytes as well as mouse erythroleukemia cells (13, 14), a variety of experiments failed to demonstrate a role for hnRNP A1 on ARE-dependent turnover or translation of reporter gene constructs or GM-CSF mRNA. We concluded therefore that another AUBP besides hnRNP A1 mediates ARE-dependent gene regulation. Experiments to identify the trans-acting factor responsible for mediating the rapid turnover of GM-CSF message were undertaken. Despite the absence of hnRNP A1, CB3 cytosols contain a 36-kDa protein that binds specifically to the ARE of GM-CSF RNA. All previously characterized AUBP of comparable size (GAPDH, HuR, and AUF1) were excluded using immunoprecipitation and two-dimensional NEPHGE immunoblotting approaches.

Matrix-assisted laser desorption/ionization-mass spectrometry identified the p36 AUBP as LDH. This finding was confirmed by immunoprecipitation of LDH-GM-CSF RNA com-
plexes from CB3 cytosols as well as by in vitro binding (UV cross-linking and filter binding) assays with purified LDH. In addition, incubation of either LDH or CB3 cytosol (data not shown) with increasing concentrations of NADH/H+ inhibited p36 AUBP activity. This latter finding is consistent with the interpretation that the Rossmann fold serves as the RNA binding domain of LDH, as has been reported previously (20) for other glycolytic enzymes. LDH localized to the polysomes in multiple cell types besides CB3 cells. Thus, the polysomal location of LDH did not occur due to the absence of hnRNP A1. Moreover, in all cell types examined, LDH localized to the bottom of the sucrose gradients, consistent with its association with mRNA being actively translated. In addition, direct association of LDH with AUF1 was demonstrated.

The role of LDH in intermediary metabolism has been well documented. LDH is a tetrameric enzyme with five isoforms, each consisting of combinations of two subunits, LDH-M and LDH-H (17). The M subunit catalyzes the conversion of pyruvate to lactate under anaerobic conditions, whereas the H subunit kinetically favors the conversion of lactate to pyruvate and predominates in aerobic tissue, such as heart muscle (17). The biosynthesis of each subunit and thus the amount of each LDH isozyme in a given tissue type is subject to genetic regulation. Although each isozyme catalyzes the same reaction, they have markedly different $K_m$ values for pyruvate (17). The isozyme identified in these studies consists of four identical M subunits and is therefore designated M4. This isozyme predominates in skeletal muscle and has a low $K_m$ for pyruvate; hence it readily transfers electrons from lactate to NADH/H+, yielding pyruvate and NADH (17). This reaction is dependent on the binding of NADH to LDH in the Rossmann fold (17).

Other enzymatic proteins have been identified that bind RNA; these RNA binding activities appear distinct from their functions in metabolism (reviewed in Ref. 27). In previous work from this laboratory (20), GAPDH was purified as a polysomal p36 AUBP from human spleen. A number of features of LDH led us to conclude that LDH and GAPDH play differing roles in post-transcriptional gene regulation. First, GAPDH appeared to be less specific in its interaction with ARE than LDH, as it
was shown to bind a variety of ARE, including those lacking reiterated AUUUu pentamers (IFN-γ and c-Myc) in their 3′-UTR (20). Second, nitrocellulose filter binding experiments performed with purified protein revealed the $K_D$ of GAPDH for GM-CSF RNA is approximately twice that of LDH for GM-CSF RNA (data not shown). These data indicate that although GAPDH and LDH are capable of binding ARE, LDH binds the 3′-UTR of GM-CSF RNA with greater affinity than GAPDH when analyzed as a purified protein. The interaction of LDH in vivo with other proteins may further enhance this difference in affinity, as we were unable to detect any GM-CSF RNA binding by GAPDH in CB3 cytosols, despite operating under conditions of probe excess. In this regard, immunoprecipitation studies demonstrate that LDH, but not GAPDH, interacts with AUFI in vivo (data not shown). These data reflect the specificity of the LDH/AUFI association, as well as suggest that LDH and GAPDH may play different roles in ARE-dependent gene regulation, with distinct substrate specificity and function.

The localization of LDH to the polysomes of human lymphoid and monocytic cells, which express GM-CSF, supports a functional role for LDH in the regulation of gene expression in vivo. The functional relevance of LDH AUBP activity is supported by its specific interaction with mRNA undergoing active translation. As levels of LDH on polysomes were approximately one-tenth those in the S10 fraction across cell types and species (data not shown), levels of polysomal and cytosolic LDH appear to be tightly regulated. Interestingly, the NAD$^+$ concentration (1 μM) required to significantly inhibit RNA binding by purified LDH is lower than cytosolic NAD$^+$ levels (30–70 μM) (32, 33). Displacement of LDH from the polysome compartment required much higher NAD$^+$ concentrations (100 μM) than those necessary to inhibit RNA binding by purified enzyme. These data suggest that polysomal LDH may have an enhanced affinity for RNA or a decreased affinity for NAD$^+$, perhaps mediated through post-translational modification or protein-protein interactions.

In this regard, we have shown that polysomal LDH exists in a large protein complex in the absence of RNA binding. By immunoprecipitation, we demonstrated AUFI and LDH exist as a complex in vivo. This finding is consistent with the sedimentation of LDH in the most dense gradient of the polysomes, indicating active translation, because AUFI exists in a complex including heat shock proteins hsp-70, translation initiation factor eIF4G, and poly(A)-binding protein (31). In support of this model is our finding that hsp-70 communoprecipitates with AUFI and LDH in THP-1 monocytic cells (Fig. 7A).

These data are especially intriguing in light of the diverse cellular functions which have been described for AUFI, which range from telomere maintenance to transcription, as well as mRNA turnover (9–12, 34). In the K562 erythroleukemia cell line, distinct effects on mRNA stability have been noted. Kiledjian et al. (11) demonstrated that AUFI is a component of the α-globin mRNA stabilization complex. Similar to our findings with hnRNP A1, overexpression of AUFI in the K562 erythroleukemia cell line had no effect on ARE-mediated mRNA turnover (35). However, when induced by hemin to undergo erythroid differentiation, ARE-mediated turnover in K562 erythroleukemia cells was inhibited. Overexpression of AUFI led to a restoration of ARE-mediated turnover (35). It is unclear if the changes in ARE-dependent turnover induced by hemin treatment or AUFI were due to effects on translation and loading onto polysomes. This observation is potentially important as ARE-mediated turnover has been reported to be dependent on either translation or ribosomal transit (36). Thus, it is possible that overexpression of AUFI restored ARE-dependent mRNA turnover by permitting polysomal loading of these mRNA.

These studies are central to consideration of the functional role of the ARE, as protein-protein interactions may influence the role(s) AUFI serves in DNA and RNA metabolism. In this regard, our data indicate that LDH is associated with AUFI on mRNA undergoing active translation. Nevertheless, LDH was initially characterized because of its potential role in mediating rapid ARE-dependent turnover in erythroleukemia cells that lack hnRNP A1. One potential model for LDH function is that it provides further specificity for the translation and turnover of certain ARE-containing mRNA through its direct interaction with both the ARE and AUFI protein. The importance of such a role by LDH is suggested by the finding that recombinant AUFI binds an array of RNA ligands, including those lacking ARE (11, 12, 34, 37). Alternatively, LDH may influence, through its direct interaction with AUFI, the makeup of this protein complex or the functional nature of its interaction with ARE. For example, a model has been proposed in which AUFI mediates ARE-dependent turnover through proteasomal targeting and degradation of the RNP complex (31). In this regard, it is important to note that LDH did not copurify with polysomes following EDTA treatment of polysomes gradients (data not shown). The possibility that LDH may serve a distinct role in mRNA translation and turnover independent of AUFI is not excluded by this model.

Additionally consistent with its association with both AUFI and hsp-70 in vivo, LDH may mediate effects on mRNA turnover as a component of eukaryotic degradation machinery, in a manner analogous to enolase in E. coli. The degradosome of E. coli consists of a high molecular weight complex of proteins including RNase E, an endoribonuclease; polyribonucleotide nucleotidyltransferase (PNPase); an ATP-dependent helicase and 3′-5′-exoribonuclease; RhlB, a member of the DEAD box family; polyphosphate kinase; and enolase, a glycolytic enzyme (38). Additional proteins are often associated with the degradosome, notably the heat shock chaperones GroEL and DnaK (38). The metabolic function of enolase has been defined as the catalysis of 2-phosphoglycerate to phosphoenolpyruvate (17), although its role in mRNA degradation is not yet clear (39).

In conclusion, the discovery of LDH as an RNA-binding protein points to an expanded role for this protein in the regulation of gene expression. Its binding specificity, polysomal localization, and association with AUFI collectively suggest a role in ARE-dependent mRNA turnover beyond its function in metabolism. The ability of LDH to serve as an AUBP may represent a global mechanism for regulating ARE-mediated decay, perhaps by modulating the effects of AUFI. Of particular note, c-Myc overexpression results in the up-regulation of LDH at the level of gene transcription (40). Elevated levels of LDH are frequently detected in human cancers (41–44). The overexpression of LDH may thus confer neoplastic growth advantage, either through its enzymatic or gene regulatory function. Understanding the dual functions of this protein may lead to greater understanding of carcinogenesis.

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LDH is an ARE-binding protein that interacts with AUF1.