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Identification of AUF-1 ligands reveals vast diversity of early response gene mRNAs

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ABSTRACT

Cell activation is associated with diverse and widespread changes in gene expression at both the transcriptional and post-transcriptional levels. AUF1 is a recently described cytoplasmic protein which likely participates in the post-transcriptional regulation (PTR) of AU-rich (ARE) mRNAs including those coding for cytokines and proto-oncogenes. Individual mRNAs subject to AUF1-mediated PTR can be predicted if AREs are present or the mRNA in question interacts *in vitro* or *in vivo* with AUF1. However, there are few, if any, general approaches for characterizing the overall repertoire of mRNAs subject to PTR by AUF1. In an effort to identify these mRNAs, we incubated total mRNA from mitogen-activated peripheral blood mononuclear cells (PBMCs) with AUF1 *in vitro*. AUF1–mRNA complexes were retarded on membranes, bound mRNAs eluted with high salt, and either used to generate a cDNA library or rebound to AUF1 a second or third time prior to elution and cDNA library construction. We have obtained partial nucleotide sequences from 130 clones which shows that the AUF1 selected libraries are rich in mRNAs containing 3′ untranslated region AREs including a large number of early response gene cDNAs. As a test of the validity of this method, we also show that a randomly selected, novel mRNA contained in the library is stabilized upon cell activation.

INTRODUCTION

Early response genes (ERGs) are dually expressed during the early phases of cellular response to growth arrest or stimulation (1–3). ERG encoded proteins include transcriptional modulators, structural proteins and cytokines (3–7). These genes are likely essential and thus ubiquitously expressed by many different cell types including fibroblasts (6,7), tumor cell lines (2) and mitogen- or antigen-stimulated T and B lymphocytes (6,8).

The regulatory mechanisms controlling ERG expression are complex. Their rapidity of induction implies coordinated transcriptional and post-transcriptional regulation (PTR). While much research has focused on elucidating transcriptional regulation, relatively little is understood concerning the post-transcriptional control of ERG mRNAs. Sequence analysis of the 3′ untranslated region (UTR) of ERG mRNAs often reveals adenosine–uridine-rich domains. These regions are often clustered into AUUUA repeats or AU-rich elements (AREs) which are widely accepted as mRNA instability determinants (9–11). Recent work suggests that the GUUUG repeats (2,12,13) or polyuridine stretches with interspersed purines (13–16) also exhibit destabilizing activity. These data suggest that multiple, uridine-rich elements can mediate instability and do so in the context of multiple, coordinately expressed mRNAs.

The underlying molecular mechanisms for ARE-mediated decay remain unclear. ERG mRNAs are extremely unstable in resting cells, often displaying half-lives of <1 h. In activated cells, such mRNAs are stabilized, often by >10-fold. Several authors have proposed that ARE-specific mRNA binding proteins are responsible for regulating ERG mRNA decay (15,17–26). Overexpression of HuR, a mammalian homolog of the ELAV proteins, stabilized vascular endothelium growth factor (VEGF) mRNAs (24). Conversely, other mRNA binding proteins may target ARE containing mRNAs for rapid decay in resting cells. AUF1 is related to the heterogeneous nuclear ribonuclear proteins (hnRNPs) A, C and D (19,24–26) and is expressed as four related isoforms with molecular weights of 37–45 kDa (24–26). It binds specifically and with high affinity to c-*myc*, c-*fos*, GM-CSF and other ERG mRNAs (22,24,25). While the precise role for AUF1 in ERG mRNA decay remains unknown, its high binding affinity, polysomal localization and target population strongly suggest a role of AUF1 in the regulation of ERG mRNA levels.

A variety of approaches including differential display (27,28) and subtractive hybridization (29,30) have been successfully used to identify specific mRNAs subject to up- or down-regulation under particular conditions. While important, such attempts focus on single genes and ignore the diversity, complexity and potential interactiveness of the ERG repertoire. DNA/RNA microchip technology might ultimately address these questions although thorough prior knowledge of the gene targets will be obligatory. Thus, we asked if a catalog of ERG mRNAs induced upon mitogen activation and subject to PTR through interactions with AUF1 could be obtained by an alternative technology. As many ERG mRNAs contain reiterated AREs, we hypothesized that a

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high affinity ARE-specific binding protein such as AUF1 could be used to selectively isolate ERG mRNAs from a complex mix of total mRNAs. Such a collection would represent the subset of coexpressed ERG mRNAs capable of interacting with the mRNA binding protein. Conversion to cDNA and packaging into a vector would create an AUF1 ligand enriched library whose characterization would yield a snapshot of co-expressed genes and likely unknown, related genes as well. Keene and co-workers reported a similar approach utilizing Hel-N1 and mRNA from neuronal cell lines (31).

In this report, we show that recombinant AUF1 has adequate specificity and affinity for the specific isolation of target ERG mRNAs. Multiple AUF1 selected mRNAs were used to construct cDNA libraries from which approximately 130 clones have been partially analyzed. As hypothesized, the AUF1 selected library was greatly enriched with ERG mRNAs as well as a significant number of unknown clones. As AUF1 selected mRNAs should be subject to PTR, we evaluated several novel mRNAs for changes in decay rate upon cell activation and ability to bind AUF1 *in vitro*. We show that a representative, randomly selected mRNA present in an AUF1 selected cDNA library is transcriptionally up-regulated and stabilized after mitogenic stimulation of peripheral blood mononuclear cells (PBMCs). Thus, we propose that AUF1 chromatography can be used to identify candidate ERGs as well as characterize the array of mRNAs co-post-transcriptionally regulated by AUF1 in activated cells.

MATERIALS AND METHODS

Band shift assays

Recombinant AUF1 containing a six-histidine N-terminal tag was expressed in *Escherichia coli* XL-1 blue and purified to near homogeneity on a nickel chelate column using standard techniques. 100 ng of purified AUF1 was then incubated with 7 ng (1–2 × 10^5 c.p.m.) radiolabeled, *in vitro* transcribed (Message Machine Kit, Ambion) wild type (wt) or mutant (mut) GM-CSF mRNA for 10 min at 4°C in 15 mM HEPES pH 8.0, 10 mM KC1, 0.2 mM DTT, 10% glycerol and 2 µg yeast tRNA. The mutGM-CSF mRNA contains reiterated AUGUA repeats in place of AUUUA repeats (32,33). The unbound probe was then cleaved by the addition of 20 U RNase T1 (Sigma) and incubation for 30 min at 37°C. The reactions were then electrophoresed on a 7% native polyacrylamide gel and, after drying, exposed overnight to Kodak X-Omat film at –80°C with two intensifying screens.

Selection of mRNA by AUF1 affinity chromatography

To optimize AUF1–RNA interactions and recovery, *in vitro* transcribed, radiolabeled wt- and mutGM-CSF RNAs were incubated with 2 µg of AUF1 in 100 µl of binding buffer (BB1: 200 mM KoAC, 50 mM Tris–HCl pH 7.5 and 10 mM DTT) and incubated at 4°C for 30 min. The mix was then transferred to 37°C for 3 min prior to gentle filtration through a pre-wet methylcellulose filter disc (Millipore HAWP 0047) and washing with 13 ml of 4°C BB1. The filter was never allowed to dry. Retained RNA was eluted with 2 ml of 37°C BB2 (BB1 supplemented with 500 mM NaCl), and collected. For control experiments, retained and eluted radiolabeled RNA was assessed by scintillation counting. For PCR analysis of mRNAs, eluted material was precipitated, washed and resuspended in water. Twenty-five percent of the resuspended RNA was reserved for RT–PCR. The remainder was reincubated with AUF1 as described above and the filtration–elution process repeated to generate RNA populations selected one, two or three times. Equal amounts of eluent from all 3 rounds were subjected to reverse transcription (GIBCO-BRL Superscript 2, used according to the manufacturer's protocol) and PCR using GM-CSF- and amyloid protein precursor (APP)-specific primers. Products were then analyzed on 1% agarose gels.

Isolation of PBMCs and mRNA

Human PBMCs were isolated from healthy donors after phlebotomy by Ficoll-hypaque density centrifugation (32). The mononuclear cells were carefully collected and washed twice with PBS. Cells were resuspended in RPM1 1640 medium, supplemented with 10% fetal calf serum, 0.1 mM L-glutamine and penicillin–streptomycin and maintained at 37°C in 5% CO_2 at a density of 5×10^6 cells/ml. For activation, tetradecanoylphorbol-13-acetate (TPA) and phytohemagglutinin (PHA) were added to final concentrations of 20 ng/ml and 5 µg/ml, respectively. After 5 h of activation, cells were collected and lysed in TRIreagent (Molecular Research Center, Cincinnati, OH, as described by the manufacturer) and total RNA isolated. mRNA was isolated by oligo-dT chromatography as previously described (33).

Selection of mRNA through AUF1 affinity matrix

PBMC mRNA (1 µg) was incubated with 18 µg of AUF1 in 100 µl of BB1 prior to filtration and elution from methyl cellulose filters (see above). An aliquot of 13 µl of this mRNA was reserved and used to construct elution library #1. The remaining 27 µl of the first elution (from a total of 40 µl) was reincubated with 36 µg AUF1 in a 200 µl reaction volume of BB1 and the binding, filtration, washing and elution processes repeated. This second elution mRNA was resuspended in 26 µl of water with 13 µl reserved for RT–PCR and the rest incubated with 18 µg AUF1 in 100 µl of BB1. The eluted mRNA from the 3rd round was suspended in 13 µl water. Each of the 13 µl mRNA pools (elution mRNAs #1–3) were used to construct AUF1 selected cDNA libraries (designated elution libraries #1, 2 or 3).

Construction of cDNA libraries

AUF1 selected mRNA samples from the 1st, 2nd and 3rd elution rounds were diluted to 25 µl in water, heated at 65°C for 5 min, chilled on ice for 3 min and then mixed with 25 µl of oligo-dT paramagnetic beads (Dynal) in 2 µl bead binding buffer (20 mM Tris–HCl pH 7.5, 1 M LiCl, 2 mM EDTA). The mixture was gently vortexed for 20 min at room temperature prior to separation on a magnet and washing with 25 µl of 2× first strand cDNA synthesis buffer (Gibco BRL). The mixture was finally suspended in 20 µl reverse transcription buffer containing 1× first strand buffer, 10 mM DTT, 1 mM dNTP mix, 1 U RNasin, 200 U superscript II reverse transcript (Gibco BRL). After incubating the reaction mix at 37°C for 15 min, the tubes were placed at 42°C for another 45 min with occasional agitation. For the second strand synthesis, 16 µl of 10× *E.coli* ligase buffer containing 188 mM Tris–HCl pH 8.3, 906 mM KCl, 46 mM $MgCl_2$, 38 mM DTT, 1.5 mM NAD, 100 mM $(NH_4)_2SO_4$, 3 µl of 10 mM dNTP mix, 1 U *E.coli* RNase H (Boehringer Mannheim), 10 U *E.coli* ligase (Gibco BRL) was added and the final volume was brought to 160 µl with water. The mix was incubated at 16°C for 2 h with

occasional shaking. The beads were separated on a magnet and washed twice with 50 µl of 1× T4 DNA polymerase buffer containing 33 mM Tris acetate pH 7.8, 66 mM potassium acetate, 10 mM magnesium acetate and 0.5 mM DTT. The beads were then suspended in 50 µl of 1× T4 DNA polymerase buffer, 2.5 µl of 10 mM dNTP mix and 1 U of T4 DNA polymerase (Epicenter Technologies) at 16°C for 15 min and at 74°C for 5 min.

Aliquots of 70 µg of each adapter–primer (KK1 and KK2; 5′-AATACGACTCACTATAG-3′ and 5′-CTATAGTGAGTCG-TA- 3′, respectively) were diluted to 20 µl with 10× PCR buffer (500 mM KCl, 100 mM Tris–HCl pH 9.0, 1% Triton X-100), heated to 60°C and cooled to room temperature. The beads were washed twice with 50 µl of ligation buffer (LB) containing 25 mM Tris–HCl pH 7.6, 5 mM $MgCl_2$, 25 mM DTT and 12.5 µg/ml of BSA. Ligation of the adapter to the cDNA was done in LB containing 0.5 mM ATP, 1 mM hexamine cobalt chloride, 13% PEG, 70 µg of the adapter mix and 400 U of T4 DNA ligase (New England Biolabs) in 20 µl and incubated at 37°C for 30 min. The adapter ligated cDNA mix which was still on the oligo-dT beads was washed twice with 1× PCR buffer, and then amplified with CDI and CDII primers (5′-CUACUACUAC-UATTTTTTTTTTTTTTT-3′ and 5′-CUACUACUACUAAAT-ACGACTCACTATAG-3′, respectively) in 50 µl containing 112 and 340 ng of CDI and CDII, respectively, 0.2 mM dNTP mix and 2.5 U of *Taq* DNA polymerase (Fisher). The temperature profile was 94°C for 3 min, 72°C for 10 min, followed by 30 cycles of 94°C (for 30 s), 50°C (for 30 s) and 72°C (for 45 s). The last elongation step was increased to 10 min.

For gene-specific amplification, 1 µl of this PCR mix was diluted to 100 µl with 5 µl used for the amplification of GM-CSF with primers (5′-CTGGCCGGTCTCACTCCTGGACTG-3′, 5′-CAGGGCCTGCGCGGCAGCCT-3′) TNFα (5′-GCACAG-TGAAGTGCTGGCAACCA-3′, 5′-GGAAGGCCTAAGGTC-CCACTTG-3′), IL-2 (5′-CCTCTGGAGGAAGTGCTAAAT-3′, 5′-ACCATTTTAGAGCCCCTAGGGC-3′) and APP (5′-CCATT-GCTTCACTACCCAT-3′, 5′-GTACACAAAACCCATTAAT-3′). The temperature profile was 94°C for 3 min followed by 30 cycles of 94°C (for 30 s), 50°C (for 30 s) and 72°C (for 45 s), with the final elongation at 72°C for 10 min. PCR products were visualized on 1% agarose gels.

Using a magnet, the beads and supernatant were separated. An aliquot of 5 µl of this supernatant was annealed to linearized pAMP10 (Gibco BRL) using the manufacturer's protocol. The annealed product was precipitated, washed and resuspended in 20 µl TE. Aliquots of 1–5 µl were used to transform *E.coli* (XL-1 blue). Individual colonies were analyzed for inserts by PCR using 300 ng each of the M13 reverse and forward primer. Positives were grown, sequenced and searched against the GenBank and the EST databases. Sequencing was either automated (Applied Biosystems 373 sequencer) or manual with Sequenase version 2.0 (US Biochemicals).

Decay of SB#1097 mRNA in activated and resting PBMCs

PBMCs were isolated as described above and activated with 20 ng/ml TPA and 5 µg/ml PHA for 5 h prior to the addition of DRB (5,6-dichloro-1-β-D-ribofuranosyl benzimidazole) to a final concentration of 20 µg/ml. Fifteen minutes afterwards, cells were collected at time 0 and at 1, 2, 3 and 4 h intervals thereafter. RNA was isolated with TRIreagent. For northern analysis the RNA was run on 1% denaturing formaldehyde–agarose gels prior to vacuum transfer to nylon membranes (Magna, Fisher) and fixed by baking for 30 min at 65°C. Membranes were prehybridized in Quick-hyb (Stratagene) for 1 h at 68°C and then hybridized for 2 h with 2–5 × 10^6 c.p.m./ml of random primed cDNA (Prime it Kit, Stratagene). Blots were washed twice for 15 min at room temperature with 2× SCC, 0.1% SDS and once at 50°C with 0.1× SSC, 0.1% SDS for 5–15 min prior to autoradiography or phosphorimaging.

RESULTS

Binding specificity of recombinant AUF1

Recombinant AUF1 was purified under native conditions and extensively dialyzed in band shift buffer (BB1) and subjected to RNA mobility-shift assay on native polyacrylamide gels. An RNA–protein complex was generated with radiolabeled wtGM-CSF RNA (Fig. 1, left panel) while none was observed with radiolabeled, mutGM-CSF mRNA containing AUGUA (mutGM-CSF) rather than AUUUA repeats (not shown). Complexes were formed despite the presence of 200 ng/µl of yeast tRNA (~40-fold molar excess over GM-CSF probe) included to eliminate non-specific probe RNA–protein interactions. The complexes were destroyed by preincubation of AUF1 with proteinase K (not shown) demonstrating they contained both radiolabeled RNA and AUF1. The binding specificity of AUF1 was further tested by the addition of unlabeled competitor RNAs. wtGM-CSF mRNA progressively inhibited complex formation which was significantly decreased by a 2.5-fold molar excess and essentially abolished with >25-fold molar excess. Similar concentrations of unlabeled β-globin (not shown) or mutGM-CSF mRNA had little effect (Fig. 1, right panel). These data show that AUF1 binds specifically to the 3′ UTR AUUUA motifs present in GM-CSF mRNA. As binding occurred in the presence of a vast excess of tRNA, these data suggest that AUF1 may have adequate specificity and affinity to recognize AUUUA containing mRNAs in vast excesses of irrelevant mRNAs.

We evaluated the latter possibility directly by adding increasing amounts of mRNA from murine hepatocytes to the mobility shift reactions. In the presence of up to 180 ng competitor mRNA, ~50% of the AUF1–wtGM-CSF RNA shift was retained (Fig. 2). It is important to note that this source of mRNA likely contains AUUUA mRNAs which could specifically interact with AUF1. As ERG mRNAs are often present at 0.01–0.1% of total cellular mRNA in activated cells, these data suggest that, *in vitro*, AUF1 could specifically bind to AUUUA RNA ligands despite the presence of a substantial excess of irrelevant mRNAs.

Separation and retention of AUF1–GM-CSF mRNA complex on methylcellulose filters

In order to use AUF1 as an affinity matrix, we sought a simple method to partition free mRNA from AUF1–mRNA complexes. As AUF1 had a six-histidine N-terminal tag, we tried to capture AUF1–mRNA complexes on nickel chelate columns and optimize elution conditions using radiolabeled wt- and mutGM-CSF mRNAs. However, free mRNAs bound the matrix and could not be removed (data not shown). Thus, we turned to filter-based separation methods. Briefly, wt- and mutGM-CSF mRNAs were radiolabeled and incubated with recombinant AUF1 in BB1 in the presence of 300-fold molar excess tRNA or 480-fold molar excess hepatocyte mRNA. We calculated the molar excess of

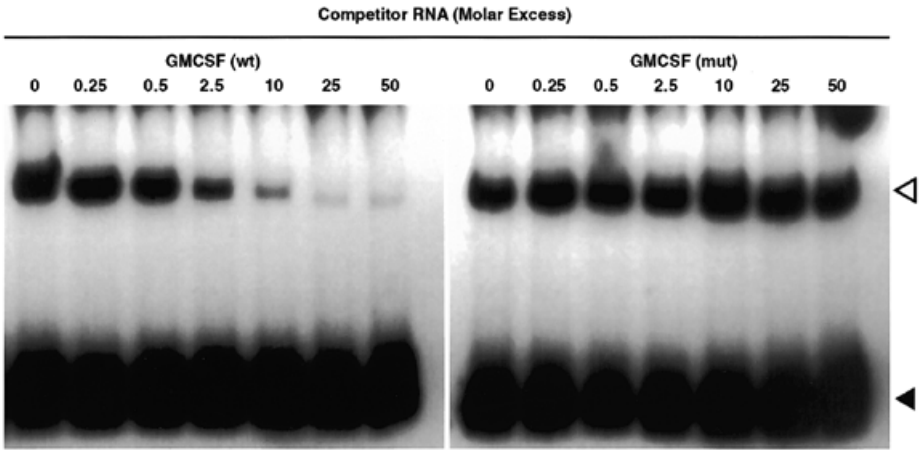

Figure 1. AUF1 specifically binds GM-CSF mRNA. Radiolabeled GM-CSF mRNA (10^5 c.p.m., 3 ng) was incubated with recombinant AUF1 (100 ng) for 10 min at 4°C in 10 mM HEPES, pH 8, 15 mM KCl, 10% glycerol and 2 µg tRNA in a total volume of 10 µl. Unlabeled competitor mRNAs (wtGM-CSF or mutGM-CSF) were added at the molar ratio shown along the top at the same time as radiolabeled probe. RNase T1 (20 U) was then added for 30 min at 37°C prior to electrophoresis on 7% native polyacrylamide gels. (**Left**) Competition with increasing amounts of wtGM-CSF RNA; (**right**) competition with increasing amounts of mutGM-CSF RNA. Specific complexes are shown by the open arrows along the right side while free radiolabeled GM-CSF mRNA is denoted by the closed arrow.

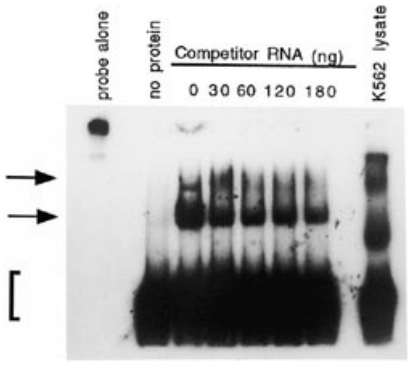

Figure 2. Irrelevant mRNA fails to block AUF1–GM-CSF mRNA complex formation. Radiolabeled GM-CSF mRNA (10^5 c.p.m., 3 ng) was incubated with recombinant AUF1 (100 ng) for 10 min at 4°C in 10 mM HEPES, pH 8, 15 mM KCl, 10% glycerol, and increasing amounts of hepatocyte mRNA (as shown along the top). RNase T1 (20 U) was then added for 30 min at 37°C prior to electrophoresis on 7% native polyacrylamide gels. Specific complexes are shown by the solid arrowheads along the left while free RNA is denoted by the bracket. K562 cytoplasmic lysate serves as a positive GM-CSF RNA mobility shift control.

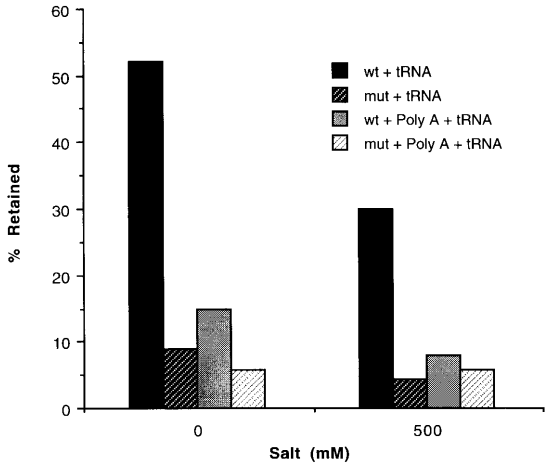

Figure 3. AUF1–GM-CSF mRNA complexes are retained by methylcellulose filters. *In vitro* transcribed, radiolabeled wt- or mutGM-CSF RNAs (darker and lighter shaded columns, respectively; ~3 ng each) were incubated with 2 µg AUF1 and 100-fold molar excess tRNA or 120-fold molar excess murine mRNA in bead binding buffer for 10 min at 4°C. The mix was then filtered through a Millipore methylcellulose filter under gentle vacuum, washed with >10 ml binding buffer without or with 500 mM NaCl and scintillation counted.

hepatocyte mRNA by assuming an average message size of 1.5 kb. The mix was then filtered through a methylcellulose filter and washed with 1200-fold excess (by volume) of BB1. The filter-bound radioactivity was assessed by scintillation counting (Fig. 3). 8–12-fold more wtGM-CSF than mutGM-CSF was consistently retained in the presence of excess yeast tRNA non-specific competitor which decreased to 3–5-fold more wtGM-CSF in the presence of excess hepatocyte mRNA. We believe these differences result from AUUUA containing AUF1 ligands present in the competitor, hepatocyte mRNA pool. We also noted the filter bound radioactivity could be eluted with 500 mM NaCl. The integrity of the eluted RNA was verified by polyacrylamide gel electrophoresis which showed a near identical size distribution as the original mRNA prior to selection (data not shown). Thus these data suggested that specific AUF1–GM-CSF mRNA complexes can be selectively retained by the methylcellulose filter and disassociated with elevated salt concentrations.

Having established conditions to reversibly capture protein–mRNA complexes, we next tested whether AUF1 could select GM-CSF from a complex pool of mRNA and whether additional rounds of repetitive selection, washing and elution could further enrich the final yield of GM-CSF mRNA. In activated lymphocytes, ERG mRNAs can accumulate to ≥1/1000 of total cell mRNA (3). In order to mimic these conditions, we spiked 200 ng of hepatocyte mRNA with 200 pg of *in vitro* transcribed GM-CSF mRNA. GM-CSF mRNA was not present in the murine hepatocyte mRNA used (based on northern blotting; data not

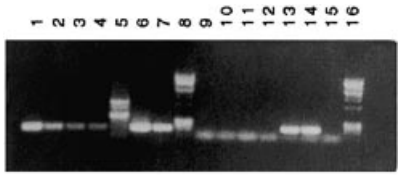

Figure 4. AUF1 affinity chromatography selectively retains GM-CSF mRNA. 200 pg of GM-CSF mRNA was mixed with 200 ng hepatocyte mRNA and incubated with 2 µg AUF1 in binding buffer at 4°C for 15 min. Mixtures were filtered through methylcellulose, washed extensively with binding buffer and specific mRNA eluted at 37°C with binding buffer supplemented with 500 mM NaCl. One-quarter of the eluted material was reserved with the remainder precipitated, resuspended in binding buffer and rebound to 2 µg AUF1. This process was repeated four times. An aliquot of each elution was subjected to RT–PCR with either GM-CSF-specific primers or APP primers prior to electrophoresis on a 1% agarose gel. Lanes 1–7, PCR with GM-CSF primers (lanes 1–4 correspond to elutions 1–4); lane 5, RT–PCR of hepatocyte mRNA starting material before the addition of exogenous GM-CSF mRNA; lane 6, RT–PCR of spiked starting material (200 pg GM-CSF in 200 ng hepatocyte mRNA); lane 7, RT–PCR of *in vitro* transcribed GM-CSF mRNA (positive control); lane 8, molecular weight markers; lanes 9–12, RT–PCR with APP primers of elutions 1–4; lane 13, RT–PCR of original starting material with APP primers (200 ng hepatocyte mRNA); lane 14, RT–PCR with APP primers of spiked starting material (200 pg of *in vitro* transcribed GM-CSF mRNA in 200 ng hepatocyte mRNA); lane 15, RT–PCR of *in vitro* transcribed GM-CSF mRNA with APP primers; lane 16, molecular weight markers.

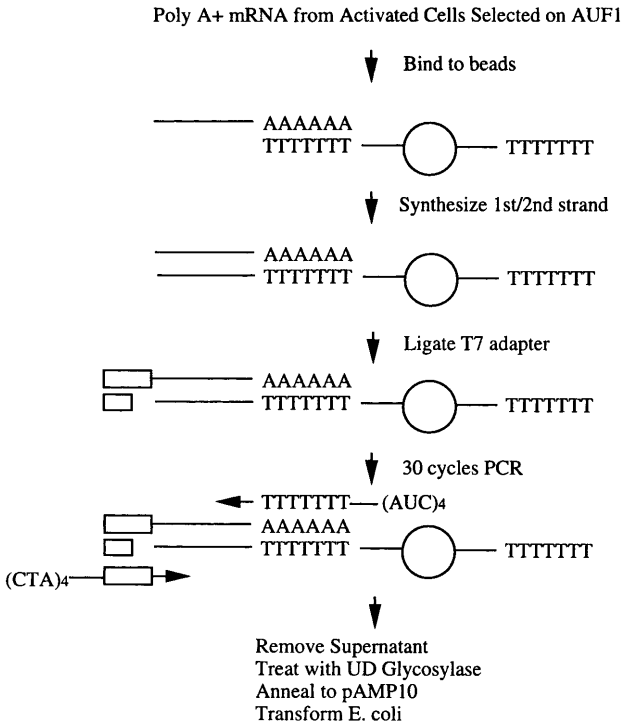

Figure 5. Schematic flow sheet for construction and amplification of micro cDNA library.

shown). The mRNA pool was then incubated with 2 µg of AUF1 as above. The mix was filtered through methylcellulose filters and the bound mRNA eluted, precipitated and resuspended again with AUF1 for a total of 4 rounds. Theoretically, this should progressively enrich the pool towards ARE mRNAs. An equal aliquot from each round was subjected to RT–PCR with GM-CSF- and APP-specific primers. The latter lacks AREs, fails to bind to AUF1 in RNA mobility shift assays (data not shown), but is present in the initial hepatocyte mRNA pool (Fig. 4, lane 13). Figure 4 shows that the initial combined mRNA pool was positive for both GM-CSF and APP (lanes 6 and 13) while the initial hepatocyte mRNA pool was negative for GM-CSF mRNA (lane 5; note the lack of a specific band despite the presence of non-specific PCR products). GM-CSF was retained in all four eluted pools of mRNA (Fig. 4, lanes 1–4) while APP mRNA was lost in the first selection (Fig. 4, lanes 9–12). A faster migrating band was commonly seen in lanes containing APP-specific RT–PCR products. Based on size, it likely represents primer–dimers. The amount of GM-CSF mRNA was slightly decreased from cycle 2 to 4. Thus reiterative selection and elution retains specific mRNA at the expense of non-specific APP mRNA and strongly suggests that affinity chromatography can be used to isolate AUF1 mRNAs ligands induced on cell activation.

Construction of a cDNA library from mRNA eluted by AUF1 affinity chromatography

mRNA was isolated from activated PBMCs treated with TPA and PHA for 5 h. As an activation control, the starting mRNA was subjected to RT–PCR which showed the presence of GM-CSF, TNFα and IL-2 (data not shown). The pool of target mRNA was subjected to 3 rounds of AUF1-binding/elution with an aliquot from each round reserved for the construction of magnetic bead-based cDNA libraries. A schematic of the protocol is shown in Figure 5. Aliquots from each of the PCR-amplified cDNA libraries were diluted and amplified with specific primers for GM-CSF, TNFα, IL-2 and APP. The first three are activation-specific ERG mRNAs containing 3′ UTR AREs, previously shown to be AUF1 ligands. APP again served as a negative control. As shown in Figure 6, all three cytokine mRNAs were present in all cDNA libraries whereas APP was absent. Again primer–dimer formation was present in samples subjected to PCR with GM-CSF, TNF and APP primers but specific, appropriate sized amplification products were only present with the former two primer sets. These data show that AUF1 selection is capable of retaining several ARE containing mRNAs without significant contamination by irrelevant species. Secondly, as seen in Figures 4 and 6, ARE mRNAs were well retained through successive cycles of selection, permitting effective reduction of irrelevant mRNAs.

An aliquot from the PCR amplified product from the 1st and 3rd round was treated with UDGase and annealed to pAMP10 and the mix electroporated into *E.coli* with Xgal/IPTG. After plating, approximately 1000 white colonies were present. Random colonies were picked and analyzed for inserts by PCR. Approximately 20% of the white colonies contained recombinants and were sequenced. The sequence was compared to GenBank and the EST databases by the Blast search tool. To date, 130 clones have been isolated and partially sequenced from the 3rd elution library and are shown in Table 1. As can be seen, the majority of mRNAs in the library are ERGs. In some cases, the same gene is present in multiple copies (p21, IL-8). This may represent greater abundance in the initial mRNA pool or higher binding affinity to AUF1. We have also identified cDNAs for mitochondrial tRNAs (9% of the clones) and ribosomal proteins (22% of the total clones). Many of these species are up-regulated on cell activation

and contain substantial AU- or GU-rich domains. Finally, 25% of the total clones sequenced showed no homology to the non-redundant database although, in almost all cases, homologous sequence fragments were present in the EST database, demonstrating their authenticity as human genes. To further confirm this, we performed northern blot analysis of five unknown clones and identified unambiguous signals in activated PBMC RNA in all cases (not shown). Of the cDNAs likely to be true positives, >90% contain AU- or GU-rich areas suggesting they were legitimate AUF1 ligands.

Analysis of clone #1097

In order to further verify that AUF1 selected cDNA libraries contained novel post-transcriptionally regulated genes, we randomly selected clone #1097 for analysis. Database search revealed an overlapping, nearly identical fragment in the EST database which was not present in the non-redundant database, confirming that this fragment was derived from a human gene. We next evaluated whether #1097 was a true AUF1 ligand by RNA mobility shift assay. *In vitro* transcribed radiolabeled #1097 RNA bound to AUF1 in the mobility shift assay (data not shown). Based on competition experiments, clone #1097 and GM-CSF mRNAs showed similar affinity to AUF1 (not shown).

The decay rate of clone #1097 mRNA was next assessed. We wished to compare the rate of decay of 1097 mRNA in resting versus activated PBMCs. Thus, cells were cultured with or without TPA and PHA for 5 h, prior to the addition of the transcriptional inhibitor DRB. RNA was isolated at various times and analyzed by northern blot for #1097 message. The signals were normalized to ribosomal protein RPL26 (34), to accommodate differences in loading and transfer of RNA. Figure 7A and B shows that #1097 mRNA was upregulated 4–5-fold in mitogen-treated cells as is typical for ERG mRNAs. In resting cells, #1097 mRNA decayed extremely quickly with a half-life of ~20 min which increased to 1.5 h in the presence of mitogens. Thus, #1097 mRNA is a true AUF1 ligand, and in activated PBMCs is significantly stabilized.

DISCUSSION

Mitogen-activated mammalian cells rapidly accumulate a subset of mRNAs whose protein products mediate programed responses. Such genes are induced within the first few hours of stimulation and have been collectively referred to as early response genes or ERGs. Well-known examples include proto-oncogenes like c-*fos*, c-*jun* or c-*myc*, other DNA binding proteins (3,4), steroid hormone receptors (35), transmembrane proteins (36), ribosomal proteins (37) and cytokines (3–7,38). The rapidity of ERG expression typically involves both transcriptional and post-transcriptional mechanisms. By combining increased transcription with reduced mRNA degradation, ERG mRNAs quickly accumulate, often to levels 10–100-fold higher than seen in resting cells (38). Conversely, decreased transcription and enhanced mRNA degradation rapidly quenches ERG mRNA accumulation and expression upon removal of the stimulant.

Table 1. Genes identified in the 3rd round elution library

Ribosomal proteins (23%)	ERGs (33%)	Others (9%)
S28	Gro-β	Splicing factors SC35
L4	Monocyte chemotactic protein-1	Hematopoietic proteoglycan core protein
L21	Interleukin-8	Leukophysin
L37	Cyclin-dependent kinase inhibitor p21	Capping protein α
L5	Poly A binding protein	Glutaredoxin
L41	DNA binding protein TAX REB 107	Initiation factor eIF3p36
S25	*jun-D*	hn RNP A1, A2
L9	Cell-surface glycoprotein CD44	Breast basic conserved gene BBC1
L10	B cell translocation gene BTG1	Trans-membrane protein of ER
L8	Interleukin-1 receptor antagonist	Basic transcription factor BTF2
L11	Cathepsin L8	Sn RNP core protein SmD2
S29	Purine nucleoside phosphorylase	
L39	Cyclophilin 40	
S14	Elongation factor 2	
L44	Plasminogen activator inhibitor	
S20	Elongation factor 1 alpha	
L5	T cell receptor beta chain	
L39	B94	
	Thymosin β-4	
	Ubiquitin	

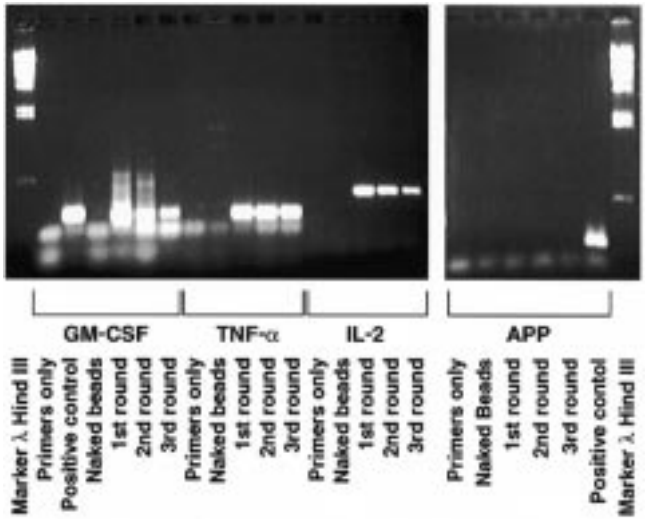

Figure 6. AUF1 eluted cDNA libraries contain multiple AUUUA-containing cytokines. cDNA libraries were produced on magnetic beads from mRNA eluted from 1, 2 or 3 successive rounds of AUF1 affinity chromatography as described above. After 30 cycles of amplification with terminal primers [$(CUA)_4$-linker and $(CUA)_4$-dT], the supernatants were removed, diluted 1:1000 and reamplified with GM-CSF-, TNFα-, IL-2- and APP-specific primers (as shown along the bottom). An aliquot of 10 µl of the PCR mix was electrophoresed on 1% agarose gels. Molecular weight markers are shown at the far right and left.

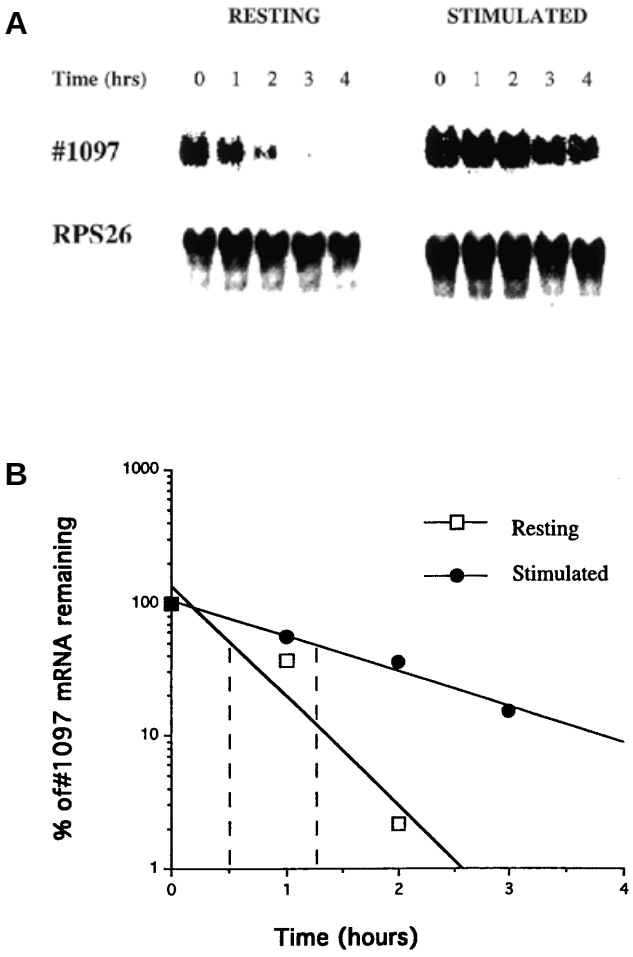

Figure 7. #1097 mRNA is stabilized and upregulated in stimulated PBMC. (**A**) PBMCs were cultured with or without TPA (20 ng/ml) and PHA (1 µg/ml) for 5 h prior to the addition of DRB. Fifteen minutes afterwards, cells were collected (time 0) and at 1, 2, 3 and 4 h thereafter total RNA was northern blotted and probed with ^{32}P-labeled #1097 cDNA (top, left and right) and ribosomal protein S26 probe (bottom, left and right). (**B**) Radioactive signals from above blots were quantified using a PhosphorImager and normalized against the S26 signal. Values are expressed as percent remaining from zero time for resting (open squares) and stimulated (closed circles) cells.

As these diverse genes show considerable co-regulation, we sought a general method to identify which ERGs would be controlled at a post-transcriptional level by AUF1. Typically, ERGs subject to post-transcriptional control have been individually identified by determining if their mRNAs decay differently in quiescence versus activated cells. The inherent instability of ERG mRNAs in resting cells usually results from 3′ UTR instability determinants of which the AU-rich element or ARE is the prototype. The ARE is a loosely defined sequence dominantly composed of uridines with occasional interspersed purines (15). The nonamer UUAUUUA(A/U)(A/U) is a potent destabilizer whose activity increased with additional copies (16). However, a destabilizing ARE lacking this nonamer has been identifed in c-*jun* RNA (39). Substitution of the adenosines with guanosines has been observed in a 3′ UTR 30-nucleotide element necessary for TGF-β-mediated stabilization of hyluronan mRNA (18). Therefore, functional AREs show considerable sequence variation making visual or computer assisted identification challenging.

Given these difficulties, we reasoned that affinity selection with an ARE-specific mRNA binding protein should yield a pool of post-transcriptionally regulated mRNAs. Several ARE-specific mRNA binding proteins have been identified and cloned (17,25). Initially identified in K562 (human erythroid leukemia) cells, AUF1 has been cloned and expressed in recombinant form (25). AUF1 contains two RNA recognition motifs (RRM), and a glutamine-rich C-terminus. The binding specificities of AUF1 have been defined and include synthetic RNAs containing $(AUUU)_4$ (25,40), the wt AREs of c-*myc* (25), c-*fos* (25), β1-adrenergic receptor (41) and GM-CSF (25) and mutant AREs of c-*myc* or c-*fos* in which the AUUUA pentamers were disrupted by single U to A point mutations (25). Thus the AUF1 recognition sequence includes ARE and ARE-related sequences with which AUF1 interacts with high affinity, making it an appropriate choice for the selection of PTR mRNAs. As expected, the 3′ UTR of AUF1 selected mRNAs show considerable diversity. While many mRNAs contain canonical AUUUA repeats (IL-8, gro-β, monocyte chemotactic protein-3, BTG-1, splicing factor SC35, DNA binding protein TAXREB107, gluteredoxin, interleukin 1-receptor antagonist and others), many contain uridine-rich 3′ UTRs with occasional purines (cathepsin B, B94, leukophysin, hematopoetic proteoglycan core protein, purine nucleoside phosphorylase, BTF-2p44, plasminogen activator inhibitor and others).

The inclusion of a particular mRNA in the AUF1 library predicts these genes are subject to PTR. Examination of Table 1 confirms this hypothesis as many of these mRNAs show variable decay in resting versus activated cells. However, we also wished to confirm that the unknown genes in the library were similarly

regulated. We randomly selected clone #1097 and showed it was a high affinity AUF1 ligand by band-shift assay. We also demonstrated that #1097 mRNA displayed considerable transcriptional and PTR in activated cells. This biological behavior mirrors that of other ERG mRNAs including GM-CSF, IL-2 and TNFα (43). Thus, we conclude that this and likely other unknown genes within the library are regulated at a post-transcriptional level.

Despite the identification of many genes through AUF1 chromatography, it remains to be shown that all are regulated by AUF1 in cells. This is especially important as the number of AU-specific mRNA binding proteins (AUBP) continues to increase. For example, the binding activity of a 30 kDa cytosolic, T-cell-specific AUBP correlates with AUUUA mRNA destabilization (43), while the activity of a 32 kDa AUBP correlates with AUUUA mRNA stabilization (11,44). Members of the Elav protein family bind AU-rich cytokine and proto-oncogene mRNAs (13,17). Hel-N1 (13), HuD (17) and HuR (45) likely play significant roles in the regultion of mRNA decay. Thus, a similar roster of cDNA clones might have been generated using another AUBP as the affinity matrix. Clone 1097 thus may be stabilized by one of the above factors, by AUF1 or a combination of AUBPs. Irrespective of the regulating AUBP, the diversity and number of probable PTR mRNAs can be appreciated from the interesting list of genes we have identified.

Despite 3 rounds of binding and elution, some possibly non-specific mRNAs were packaged into the library. Ribosomal proteins constituted a minor fraction of the AUF1 affinity selected mRNAs. These proteins range in function from translational scaffolding (46) to having anti-proliferative activity (BBC1 and L13) (47). Abnormal expression of S25, S20, L4, L5, L21, L37 and L39 have been observed during cellular transformation (48–53). However, as many of these ribosomal genes behave as true ERGs, their inclusion may be appropriate. Mitochondrial genes represent another minor fraction of the AUF1 affinity selected library. Fragmentation of the mitochondrial genome as a consequence of aging or oxidative damage and its subsequent insertion into the nuclear genome has been described (54). These rearranged genes can be transcribed, yielding authentic, chimeric mRNAs (54). The presence of AU-rich domains in many of these mRNAs as well as their identification herein by affinity chromatography suggests they may be subject to AUF1-mediated regulation *in vivo*.

The number of rounds of AUF1 selection prior to cDNA library construction were selected somewhat arbitrarily. We assumed that reiterative rounds of binding, washing and elution would enrich the pool for 'true', AUF1 selected mRNA ligands and at the same time reduce non-specific clones. These goals were confirmed by PCR and sequence analysis of randomly selected clones from the elution libraries. While we have not extensively examined the first library, the small number of sequenced genes suggests a greater percentage of ribosomal DNAs. Thus, the additional rounds of binding likely reduced the presence of extremely abundant mRNAs which may not be AUF1 ligands or are weak ligands. SELEX typically employs 5–10 cycles of binding and elution to identify the highest affinity ligands (31). Our goal, however, was to identify both low and high affinity AUF1 selected mRNA ligands rather than identify the optimal RNA sequence for binding. Thus, increasing the number of rounds would presumably reduce the number of recovered clones by progressively eliminating lower affinity ligands.

The library constructed from 3 rounds of selection has considerable complexity. PCR screens demonstrated ~20% of all white colonies contained cDNA inserts which when extrapolated, suggests that the third library contains approximately 1000 inserts. As some of these cDNAs are duplicates the true number of individual clones is somewhat smaller, probably in the range of 500–750. As 35% of the cDNAs are ERG and 25% are unknown, the library may contain as many as 100 novel cDNAs. As these genes could be bacterial, we performed northern blotting with five randomly selected, third library cDNAs against human PBMC RNA. In all cases, hybridization signals were identified after stringent washing, demonstrating the human origin of the novel cDNAs. We are presently cloning full-length versions of several novel cDNAs which based on their 3′ UTR and ability to interact with AUF1 may be proto-oncogenes, cytokines or other signaling molecules.

Finally, with modest modifications, this approach could be utilized to identify ligands for other mRNA proteins and classify their regulatory network. For example, the iron response element (IRE) confers iron-dependent, co-PTR on at least ferritin, transferrin and ALA synthase mRNAs (55). These three mRNAs bind to the IRE-binding protein (IRF; 55) which alters their stability or translation depending on the iron status of the cell (55). Whether other IRE containing mRNAs exist could be directly addressed as shown herein.

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