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Identification of proteins binding specifically to the 3′-untranslated region of granulocyte/macrophage-colony stimulating factor mRNA

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ABSTRACT

The 3′-untranslated region of granulocyte/macrophage colony-stimulating factor (GM-CSF) mRNA contributes to the post-transcriptional regulation of gene expression. Degradation is partly mediated by adenosine–uridine-rich sequence elements (ARE), which serve as binding sites for specific proteins. Stabilization of RNA by phytohemagglutinin and concanavalin A treatment is dependent on regulatory sequence elements upstream of ARE. We have performed northwestern blot and filter binding assays using cell extracts and RNA sequences containing or lacking ARE. Murine and human T cell extracts (EL-4 and Jurkat) yielded two specific proteins of 93 and 94 kDa, respectively, that were binding to sequences upstream of ARE. Within this region, the human and murine RNA do not share any obvious sequence identity, yet both are target sites for the binding proteins. The smallest RNA fragments protected by the proteins from RNase A digestion, were 44 in the murine, and 38 ribonucleotides long in the human sequence. The binding activity of the 94 kDa protein derived from human Jurkat cells could be enhanced by phytohemagglutinin. The interaction with regulatory mRNA sequences and the responsiveness to phytohemagglutinin suggests that the proteins are involved in controlling GM-CSF mRNA turnover.

INTRODUCTION

Adenosine–uridine-rich sequence elements (ARE) located within the 3′-untranslated region (3′UTR) of many cytokine, proto-oncogene and other mRNAs, mediate rapid decay. Proteins binding to such sequence elements have been identified and characterized by several laboratories (1–10). In a few cases, evidence has been presented that binding is related to a regulatory function. Destabilizing properties were attributed to the AU-binding protein AUF1 since it has been purified from post-ribosomal supernatants that selectively accelerated degradation of *c-myc* mRNA (3). A protease-sensitive factor protected interleukin-2 mRNA from rapid decay (10). The agonist induced increase of β-adrenergic receptor mRNA binding protein correlated with a destabilization of β-adrenergic receptor mRNA (11).

Concanavalin A (ConA), phytohemagglutinin (PHA), Ca^{2+} ionophore and the phorbol ester 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) lead to an increased secretion of granulocyte/macrophage colony-stimulating factor (GM-CSF) in T cells (12,13). The mechanisms leading to mRNA stabilization via pathways acting through protein kinase C, protein kinase A, tyrosine kinase or intracellular calcium release are greatly unknown. We have studied protein kinase C- and Ca^{2+}-mediated stabilization of murine GM-CSF mRNA in T cells (12,14,15). Functional data obtained for the post-transcriptional regulation of murine GM-CSF expression, suggested that ARE-mediated decay alone is not sufficient to explain TPA- and Ca^{2+} ionophore-mediated mRNA stabilization. Complete decay of a chimeric chloramphenicol-acetyltransferase-GM-CSF mRNA required more sequence elements than the cluster of AU-boxes located in the downstream half of the GM-CSF 3′UTR. TPA-mediated stabilization of this chimeric mRNA had an absolute requirement for sequences located in the upstream half of the 3′UTR. Analysis of potential sequence-specific RNA binding proteins to ARE of GM-CSF by either mobility-shift or label-transfer experiments revealed a set of RNA–protein complexes in the range of 33–42 kDa (1). These proteins specifically recognized a 63 nt cluster of AUUUA motifs located in the 3′ half of the murine and human GM-CSF 3′UTR. These RNA–protein interactions, however, cannot explain the TPA-mediated stabilization of the mRNA. Using a filter binding assay and northwestern blotting techniques, we have now detected a 93 and a 94 kDa protein in murine and human T cells, respectively. These proteins bound specifically to the sequence upstream of ARE. The activity of the 94 kDa protein was enhanced in PHA-treated Jurkat cells. These observations suggest that the proteins are involved in the regulation of GM-CSF mRNA turnover together with other cell-specific factors.

MATERIALS AND METHODS

Reagents

Reagents were purchased as follows: BSA and HEPES, Fluka (Buchs SG, Switzerland); FBS, Amimed (Muttenz, Switzerland); molecular mass standards, BioRad (Glattbrugg, Switzerland);

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MultiScreen membrane NP 0.2 µm, Millipore (Volketswil, Switzerland); nitrocellulose membrane 0.45 µm, Schleicher & Schuell (Riehen, Switzerland); penicillin and streptomycin, Life Technologies (Basel, Switzerland); PHA was isolated from soybeans; poly(I), Pharmacia (Dübendorf, Switzerland); RNase A, RNase T1, yeast tRNA and SP6 RNA polymerase from Boehringer Mannheim (Rotkreuz, Switzerland); RNase inhibitor (RNasin), Promega (Wallisellen, Switzerland); tissue culture RPMI 1640 medium, Inselspital Apotheke (Bern, Switzerland); [α-^{32}P]UTP (29.6 TBq, 800 Ci/mmol), Du Pont-New England Nuclear (Regensdorf, Switzerland).

Cell cultures and protein extracts

All cell cultures were cultivated in a 5% CO_2 incubator at 37°C. The murine thymoma cell line EL-4 (16) and the human T cell lymphoma Jurkat (17) were grown in RPMI 1640 medium supplemented with 10 % FBS, 2 mmol/l glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. For stimulation, cells were washed with PBS and resuspended at a density of 2×10^6 cells/ml in serum free RPMI 1640 medium. PHA was added to obtain a concentration of 5 µg/ml. After the indicated period, cellular metabolism was blocked on ice, cells were washed with ice-cold PBS, and proteins were extracted. Cytoplasmic and nuclear extracts were prepared according to methods described previously (18). Cells were washed with PBS and kept on ice for 10 min in a buffer containing 25 mmol/l Tris–HCl, pH 7.4, 40 mmol/l KCl, 1% Triton X-100, 10 µg/ml leupeptin and 0.12 mmol/l PMSF. A pre-chilled glass Dounce homogenizer was used to lyse the cells and nuclei were spun down at 3000 *g*. The supernatant, designated the cytoplasmic extract, was centrifuged at 30 000 *g* at 4°C for 1 h to remove debris. The nuclear pellet was resuspended in a buffer containing 25 mmol/l Tris–HCl, pH 7.4, 10 mmol/l KCl, 1 mmol/l $MgCl_2$, 275 mmol/l NaCl, 20% (v/v) glycerol, 10 µg/ml leupeptin, 0.12 mmol/l PMSF and 0.5 mmol/l DTT. After two rounds of vortexing and incubation on ice, the nuclear fraction was centrifuged at 18 000 *g* for 1 min and the precipitate was discarded. Protein concentrations were measured by standard protein assays. Lysates were stored in aliquots at –70°C until used.

Plasmid constructs and *in vitro* RNA synthesis

Sequences were derived from murine and human GM-CSF cDNAs [Loci: MMGMCSF (19) and HSCSFGM (20), respectively] and obtained by PCR amplification of plasmids pJL4GM5′Δ19 (21) and p9023 (20) with primers that included appropriate restriction sites. Amplicons were ligated into the transcription vector pSP64poly(A) (Promega). Murine sequence inserts from position 471 to 774, 471 to 658 and 497 to 556 were generated to construct the plasmids pM23, pM21 and pM41, respectively. The transcribed sequences thus contain either the entire murine GM-CSF 3′UTR (muUTR), the 3′UTR lacking the ARE (murine upstream regulatory elements, muURE) or the previously defined TPA-response element (TRE). From the human sequence, an insert from position 444 to 635 was generated to produce the plasmid pM31. This sequence (huURE) corresponds to the murine counterpart pM21. Plasmids were linearized upstream of the poly(A) stretch and transcribed *in vitro* using SP6 polymerase and a standard transcription protocol. For the synthesis of an unspecific competitor riboprobe, the original pSP64poly(A) vector with the intact multiple cloning site was linearized downstream of the poly(A) sequence. Transcripts were gel purified on 6% polyacrylamide–8 M urea gels. Competitor RNA was produced by standard large scale synthesis (RiboMAX™, Promega).

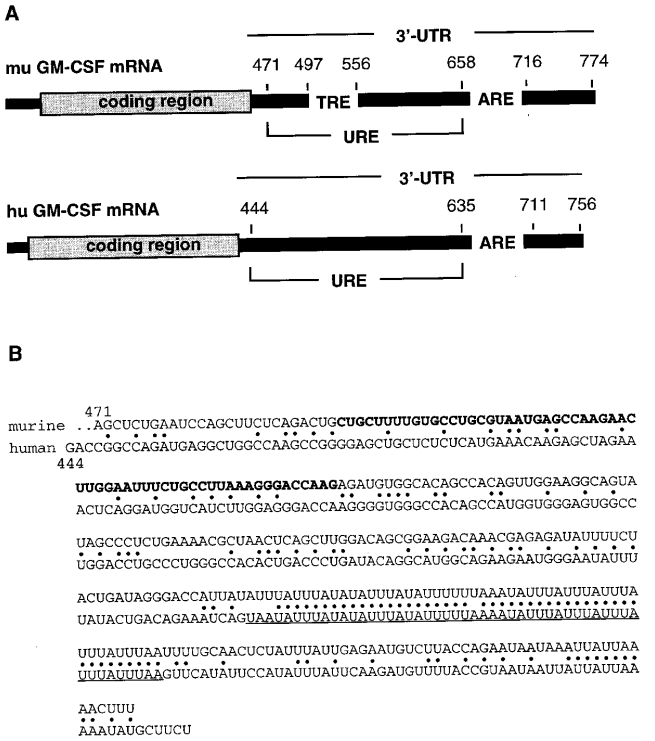

Figure 1. Schematic diagram and aligned ribonucleotide sequences of murine and human GM-CSF mRNA. Nucleotide numbers refer to GenBank accession number X03221 for the murine and M10663 for the human sequence. AU-rich sequence **e**lements (ARE, underlined) and upstream regulatory sequence elements (URE) are indicated in the murine and human 3′UTRs. In the murine sequence, a 60 nt long functional phorbolester (TPA)-response element (TRE, bold-face) lies within the URE.

Northwestern blotting

Cytoplasmic protein extracts were size separated by 12 or 7.5% denaturing SDS–PAGE and electrotransferred to nitrocellulose membranes (BA85, Schleicher & Schuell). Membrane-bound proteins were renatured overnight at 20°C in a buffer containing 10 mmol/l HEPES, pH 7.9, 40 mmol/l KCl, 5% glycerol, 0.2% Nonidet-P40, 3 mmol/l $MgCl_2$, 0.1 mmol/l EDTA, 1 mmol/l DTT and 5 mg/ml BSA. Membranes were transferred to RNA binding buffer (10 mmol/l HEPES, pH 7.9, 150 mmol/l KCl, 5 mmol/l $MgCl_2$, 8% glycerol, 0.2 mmol/l DTT, 50 µg/ml tRNA), [α-^{32}P]UTP-labeled RNA probe (6×10^6 c.p.m. for 3′UTR, 3×10^6 c.p.m. for URE) added, and membranes incubated for 2 h at 20°C, followed by digestion with RNase A (25 µg/ml) for 20 min at 37°C. Membranes were washed twice with RNA binding buffer without DTT and tRNA. Dry membranes were exposed for 1 h to PhosphorImager (Molecular Dynamics, Inc.) screens. Digitized images are presented in Figures 2, 4–6A and 7.

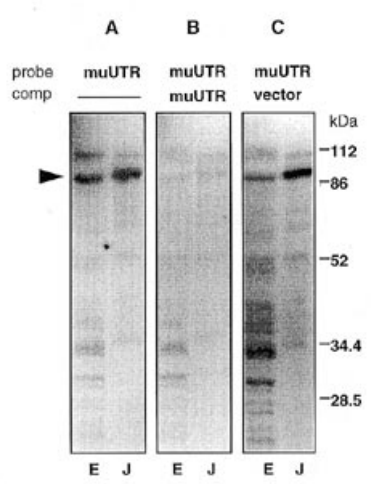

Figure 2. Northwestern blot analyses of human and murine T cell extracts. Sixty micrograms of cytoplasmic proteins from EL-4 (E) and Jurkat (J) cells were separated by 12% SDS–PAGE and electrotransferred to nitrocellulose membranes. After protein renaturation, membranes were probed with [α-^{32}P]UTP-labeled murine GM-CSF 3′UTR RNA (6×10^6 c.p.m.) in the presence of 50 µg/ml tRNA (**A**). Autoradiograph in the presence of 1000-fold molar excess of unlabeled muUTR RNA (**B**), and in the presence of vector RNA sequence containing the 50 ribonucleotide long multiple cloning site of pSP64poly(A) and a stretch of 30 adenylate residues (**C**). Molecular mass of pre-stained protein markers are indicated.

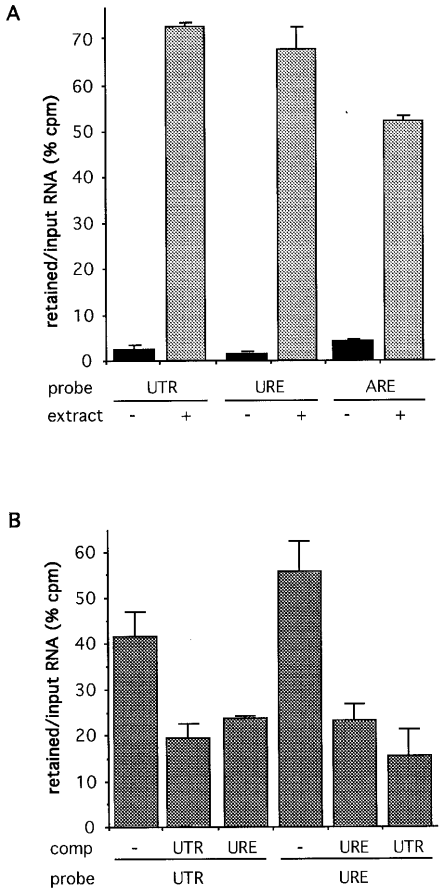

Figure 3. Detection of ARE-independent binding using a filter binding assay. Cytoplasmic proteins of EL-4 cells were incubated with radiolabeled RNA from murine GM-CSF 3′UTR, URE (i.e., the 3′ untranslated region lacking the AU-rich sequence elements) or ARE (**A**). Binding reactions were passed through nitrocellulose membranes and complexed RNA retained on the filter was determined by liquid scintillation counting. Cold competition experiments were performed with muUTR (murine 3′ untranslated region) or muURE RNA in a 10^4-fold molar excess (**B**). Data is represented as counts retained on the membrane (A) or percentage (B) of retained versus input RNA (% radioactivity). Mean values are derived from duplicate values of two independent experiments.

Filter binding assay

Cytoplasmic protein extracts were incubated for 20 min at room temperature with known amounts of *in vitro* transcribed, [α-^{32}P]UTP-labeled RNA probes (e.g. 0.06 ng = 3.6×10^{-13} mmol = 15 000 c.p.m. of muUTR per reaction, specific activity $2.9–5.8 \times 10^9$ Bq/mg) in a binding buffer containing 10 mmol/l HEPES, pH 7.6, 3 mmol/l $MgCl_2$, 5% (v/v) glycerol, 1 mmol/l DTT, 5 µg/reaction poly(I), 2 U/reaction RNasin. Reaction mixtures were transferred to prewetted nitrocellulose membranes (MultiScreen NP, Millipore) using a 96-well filtering device (MultiScreen, Millipore) and incubated at 20°C for 20 min. Filters were washed with binding buffer supplemented with 200 mmol/l NaCl, transferred to scintillation vials, and counted in a scintillation counter (LS 5000CE, Beckman, Nyon, Switzerland) by Cerenkov counting. All reactions were assayed in duplicate. Data in Figure 3 are mean values of two experiments.

Analyses of ribonuclease protected RNA fragments

Northwestern blots were treated with RNase A (25 µg/ml) for 20 min at 37°C. RNA–protein complexes localized by autoradiography were cut out and RNA extracted from the complexes with 300 µl H_2O and 200 µl phenol. After separation of the two phases 300 µl H_2O was added to the phenol phase. The pooled aqueous phases were extracted five times with 500 µl ether and their volume was reduced by vacuum evaporation. RNA was separated electrophoretically on sequencing gels together with polyribonucleotide markers of known length. Markers were generated by *in vitro* transcription in the presence of [α-^{32}P]UTP. The dry gels were exposed overnight to PhosphorImager screens.

RESULTS

Detection of specific protein interactions with the GM-CSF 3′UTR

The 3′UTR of GM-CSF mRNA has shown to be responsible for the stabilization of the mRNA by TPA, lectins, calcium ionophore A23187 and cytokines. The 3′UTR can be subdivided into two regions, the ARE and the upstream regulatory sequence element (URE) (Fig. 1A). The ARE of the human and the murine sequences are almost identical, while the UREs differ significantly (Fig. 1B). A functional TRE has been previously defined in murine GM-CSF mRNA. By performing northwestern analyses using the *in vitro* radiolabeled murine 3′UTR RNA probe, we detected a specific interaction with a 93 kDa protein from extracts of the murine thymoma EL-4 cell line, and with a 94 kDa protein from human lymphoma Jurkat cells (Fig. 2A). Addition of 1000-fold molar excess of unlabeled muUTR RNA to the

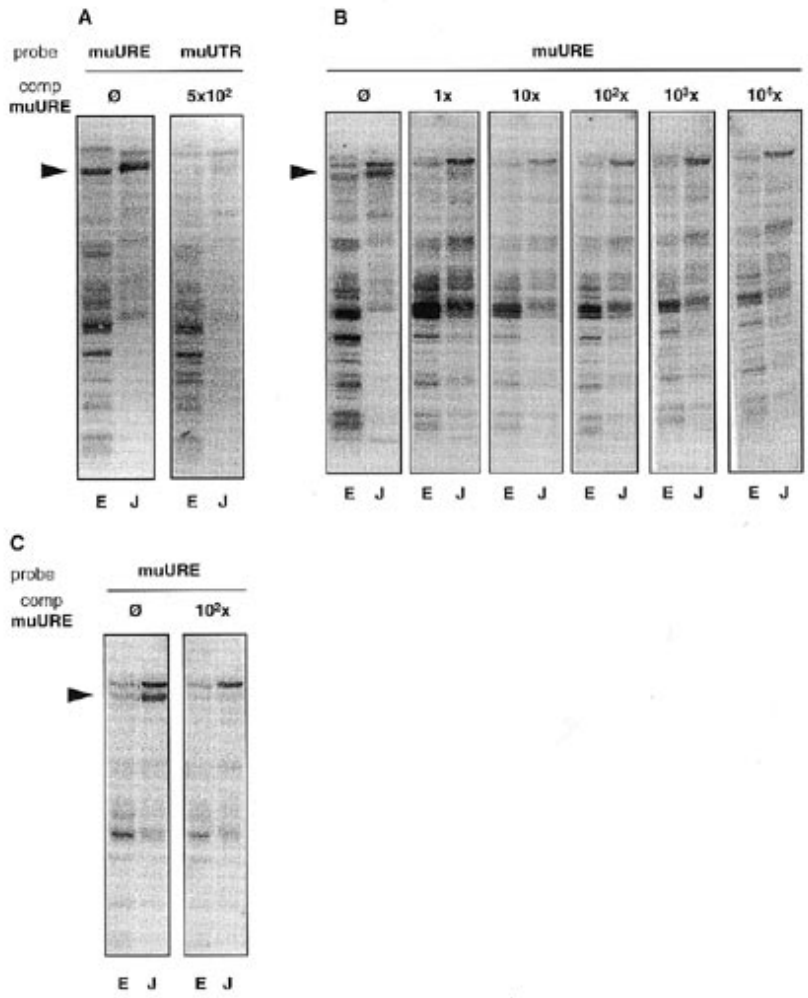

Figure 4. Competition analyses of murine sequences lacking AU-rich sequences (muURE) and murine sequences including the entire 3′UTR (muUTR) binding to murine EL-4 (E) and human Jurkat (J) cell proteins on northwestern blots. Sixty micrograms of cytoplasmic protein extracts were incubated with either radiolabeled muURE alone or in the presence of unlabeled muURE to compete binding of muUTR (**A**). Northwestern blots were incubated with radiolabeled muURE in presence of increasing amounts of unlabeled muURE as competitor (**B**). Competition of the binding reaction with unlabeled RNA added 30 min after addition of the radioactive muURE (**C**).

radiolabeled murine 3′UTR probe, almost completely abolished the signals of the two proteins (Fig. 2B). In contrast, competition with unlabeled vector sequence including a stretch of 30 adenylate residues did not significantly affect binding of the two proteins (Fig. 2C) suggesting that a specific interaction with muUTR RNA had occurred. When cell extracts treated with proteinase K were probed, no signals were obtained (data not shown).

Protein interaction is independent of ARE binding

To determine the site of the protein interactions within the 3′UTR, three different radiolabeled probes were used. Ten micrograms cytoplasmic proteins from EL-4 cell extracts were mixed with *in vitro* synthesized radiolabeled murine UTR, URE (3′UTR lacking ARE) or ARE RNA. Binding reactions were applied to nitrocellulose filters and radioactivity of complexed RNA trapped on the filters was determined (Fig. 3A). When the entire murine 3′UTR (UTR) was added to the EL-4 protein extract, 74.3% (9196 c.p.m. ± 50, mean ± s.d.) was retained on the membrane. When the probe lacking ARE (muURE) was used 68.3% (4188 ± 12 c.p.m.) was measured, while a probe with only the AU-rich sequences (ARE) yielded 52.5% (3410 ± 224 c.p.m.). Background radioactivity was highest when ARE was used (4.6%) and lowest with URE (1.7%). Subsequently, a series of competition experiments with probes including either the entire 3′UTR or the 3′UTR lacking ARE (URE) were performed in the presence of either none or unlabeled muUTR or muURE RNA (Fig. 3B). In the absence of competitor RNA, 42% of total labeled muUTR was retained, while in the presence of 10^4-fold molar excess unlabeled muUTR 20% of the label was recovered. When muURE was used as competitor instead, ~24% of the total radioactivity was recovered. Using a radioactive muURE probe, ~56% of total counts were retained on the filter. Competition with cold muURE and muUTR RNA yielded 23 and 16% of initial counts. These results show that binding of radiolabeled muUTR or muURE can be competed to about the same levels by either unlabeled muUTR or muURE. This suggests that most of the specific interaction between binding proteins and the murine 3′UTR RNA sequence may

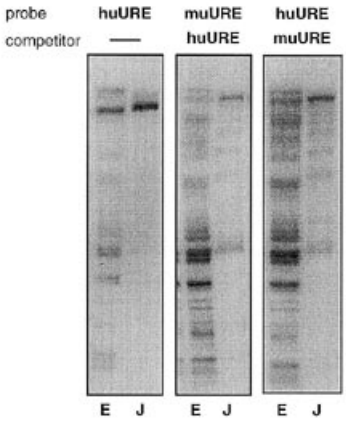

Figure 5. Murine and human RNA compete for binding to 93 and 94 kDa proteins. Cytoplasmic protein (60 µg per lane) from EL-4 (E) and Jurkat (J) cells were separated by 12% SDS–PAGE, transferred to nitrocellulose and northwestern blot analysis performed. Membranes were incubated with [α-^{32}P]UTP-labeled murine and human URE RNA. Competition was carried out with unlabeled huURE (350-fold molar excess) or muURE (200-fold molar excess).

occur in a region independent of ARE. The residual radioactivity that cannot be quenched by cold competition suggests unspecific binding of proteins to the GM-CSF mRNA 3′UTR.

Further competition reactions were analyzed by northwestern blotting. Sixty micrograms cytoplasmic protein of EL-4 or Jurkat cells was loaded per lane of a 12% SDS–PAGE, electrotransferred, and after renaturation the binding reaction carried out with radiolabeled muURE (Fig. 4A). As already observed with the muUTR probe (Fig. 2A), muURE probe yielded a similar binding pattern with the two major bands of 93 and 94 kDa. These bands were almost completely abolished in the presence of the 3′UTR probe and 500-fold molar excess unlabeled muURE. Thus, muURE is able to compete the binding of muUTR to the 93 and 94 kDa proteins in EL-4 and Jurkat cells, respectively. In contrast, other bands, especially in the 30 kDa range, were not affected by muURE competition (Fig. 4A). Using a similar experimental setup, the strength of the RNA–protein interaction was evaluated by addition of radiolabeled muURE and various concentrations of homologous cold muURE as competitor (Fig. 4B). The northwestern analysis shows that a 100-fold molar excess was needed to compete the binding completely. Again, as observed before with muUTR probe, even at a 10^4-fold-excess of cold muURE, non-specific binding to many proteins is not prevented. This most likely reflects the incomplete competition observed in the filter binding assay. In the experiments shown in Figure 4A and B, blots were preincubated for 30 min with the unlabeled competitor. In contrast, the competition experiment presented in Figure 4C was performed by adding the cold competitor RNA 30 min after the labeled RNA. Specific competitor still abolished the signal almost completely. This indicates that even after binding to the 93 and 94 kDa proteins, the labeled RNA dissociates and can be replaced by the unlabeled specific competitor.

Human and murine cross-reactivity

To investigate the presence of specific binding sites within the URE of the human GM-CSF mRNA for RNA binding protein, similar experiments as described above were carried out.

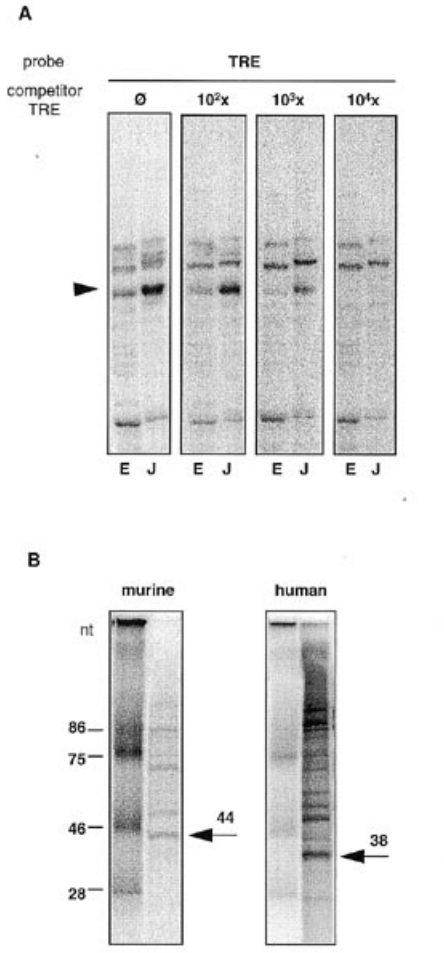

Figure 6. Characterization of the RNA binding site. Northwestern blots with cytoplasmic protein from each EL-4 (E) and Jurkat cells (J) that were separated by 7.5% SDS–PAGE and incubated with the functionally mapped 60 nt long murine phorbolester (TPA)-response element (TRE). Increasing amounts of unlabeled TRE were added as competitor (**A**). Human and murine URE RNA (3′UTR lacking ARE) protected from RNase A digestion by human and murine proteins were analyzed on a 12% sequencing gel (**B**). Synthetic polyribonucleotides of known length were used as size markers. Arrows indicate smallest protected fragments.

Northwestern blot analysis using radiolabeled huURE probe revealed an identical banding pattern as seen for muURE (Fig. 5). Competition of huURE binding to EL-4 and Jurkat cells proteins with unlabeled muURE, or vice-versa, suppressed the signals of the 93 and 94 kDa proteins (Fig. 5). In spite of any obvious identity between muURE and huURE, the two proteins seem to interact with both the murine and the human sequence.

Characterization of the RNA binding site

Northwestern analysis has provided data that the RNA binding site for the two T cell proteins lies within the 187 nt long muURE. To narrow down the binding area, a radiolabeled probe for the previously identified functional 60 ribonucleotide TRE has been used to evaluate specific binding (Figs 1 and 6A). The TRE probe bound to the 93 and 94 kDa proteins. A 10^3–10^4-fold molar excess of cold TRE had to be added to compete this binding

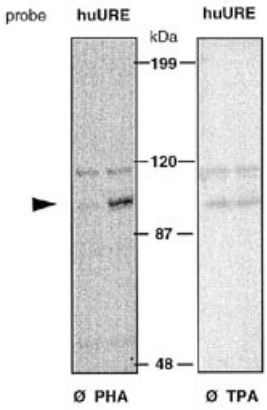

Figure 7. Up-regulation of the human 94 kDa protein–RNA interaction by PHA. Jurkat cells (2×10^6/ml) were left untreated or stimulated for 30 min with PHA, proteins extracted and analyzed by northwestern blotting (7.5% SDS–PAGE) with a radiolabeled huURE probe.

completely. Even at the highest concentration of cold competitor used, the intensity of other bands remained unaffected. To further analyze the binding area RNase A treatment of the binding complex on the northwestern blot membranes was performed. The protected fragments were recovered and separated electrophoretically on a sequencing gel. As calibrated by synthetic polyribonucleotides, the murine EL-4 and Jurkat proteins yielded smallest protected RNA fragments of 44 and 38 nt, respectively (Fig. 6B).

Regulation of the URE binding proteins

The interaction of the 93 and 94 kDa proteins within the region of the functional TRE suggests that these proteins are involved in the control of mRNA turnover. To investigate if their synthesis or binding activity can be modulated by cellular activation, the effects of TPA, ConA and PHA were tested on human Jurkat and murine EL-4 cells, murine NIH3T3 fibroblasts, human U937 promonocytes and fresh human peripheral blood mononuclear cells. Cytoplasmic and nuclear extracts from treated and untreated cells were compared by northwestern blot analyses. While no signal was seen in human peripheral blood mononuclear cells, the 93 and 94 kDa proteins were detected in all other extracts (data not shown). Unexpectedly TPA-stimulation did not induce binding activity. A clear regulatory effect, however, was observed in cytoplasmic extracts of Jurkat cells treated for 30 min with PHA. Extracts from unstimulated cells probed with huURE generated a weak signal of the 94 kDa protein, while upon PHA stimulation the signal increased significantly (Fig. 7).

DISCUSSION

The results presented here show that murine and human T cells contain a protein that binds specifically to the murine and human GM-CSF mRNA 3′UTR. Under denaturing conditions, the murine and the human protein have an apparent molecular mass of ~93 and 94 kDa, respectively. The size of the two proteins differs markedly from other RNA binding proteins characterized recently. The family of proteins with the capacity to bind with high affinity to AU-rich, and in some cases U-rich regions, fall in the range of 10–70 kDa molecules (1–3,6–10,22,23). Yet, RNA binding proteins of larger sizes have also been reported, e.g., iron regulatory proteins-1 and -2 have a molecular mass of 87 and 105 kDa, respectively (24,25). Heterogenous nuclear RNP exist with molecular masses ranging from 34 to 120 kDa, none however around 90–100 kDa (26).

Competition experiments using two types of assays and *in vitro* transcribed RNA probes have demonstrated that binding of the 93 and 94 kDa proteins to GM-CSF mRNA occurs in a region designated URE which is separated from the area containing the AREs (Figs 1, 3 and 4). While the muURE region spans ~187 nt, specific protein binding has also been observed to an RNA probe of ~60 nt in length. This area, spanning ribonucleotides 497–556 of the murine GM-CSF mRNA, is located within muURE and includes a TRE. While no such element has been characterized in the 3′UTR of human GM-CSF mRNA, both the human as well as the murine protein bound to murine TRE probe. As shown by northwestern blot assays, the binding of radiolabeled TRE could be competed by the addition of cold TRE at 10^3–10^4-fold molar excess (Fig. 6A) while a 10^5-fold molar excess of unrelated competitor (yeast tRNA) did not prevent complex formation. In contrast, URE binding was affected by 100-fold cold competitor suggesting a lower binding specificity for the partial URE probe. This difference in binding specificity may be explained by the formation of secondary and tertiary structures that are likely necessary for protein interaction (27). Computer-aided structure predictions did not reveal any similarity between the murine and human URE. Such structures may also be responsible for the cross-reactivity of the murine protein to the human URE and vice-versa, because no obvious RNA sequence homology between the two species has been observed. Furthermore, the RNA–protein interaction possibly depends on the presence of multiple binding sites. This would explain the observation that when a larger target sequence was used as a probe, the interaction was more specific. The excess rates for cold competition of labeled RNA we have used in our experiments compare to the molar excess rates of 100–500-fold reported for other specific RNA–protein interactions (23,28).

RNase A treatment of RNA–protein complexes of the 93 and 94 kDa proteins with URE yielded protected RNA fragments of 44 and 38 nt, respectively (Fig. 6B). For comparison, some murine AU-binding proteins of T cell origin protected an area of 63 ribonucleotides (1), and vitellogenin and erythropoietin mRNA binding proteins protected a region of 27 and 120 nt within their 3′UTR (29,30). While the length of the protected fragments may provide information on single- or multifactor binding, the actual site of RNA–protein interaction is certainly limited to much smaller regions.

The finding that the 93 and 94 kDa proteins interact specifically with a sequence element required for TPA- and calcium-ionophore-mediated stabilization of the mRNA, may imply a role for the two proteins in regulation of GM-CSF mRNA turnover. An upregulation of the 94 kDa protein in human Jurkat cells has been observed in response to PHA stimulation (Fig. 7). In fact, PHA has been demonstrated to induce release of three major forms of GM-CSF from human T lymphocytes (13). Therefore, PHA may act in part via enhancement of protein binding to sequence elements on huURE and thus increase GM-CSF mRNA stability. The binding process may be directed by processes such as protein phosphorylation/dephosphorylation of the binding protein. The fact that no upregulation has been observed in PHA treated murine EL-4

cells, nor in TPA-treated Jurkat cells and in a few other cell types tested, suggests cell- and stimulus-specific differences. Such cell-specific differences have also been observed for TPA-mediated stabilization of GM-CSF mRNA. While stabilization was observed in murine EL-4 cells, no such effect was detected in murine fibroblasts (14). In addition, GM-CSF mRNA is stable in certain tumor cell lines whereas in some others it is not (31). These cell type-specific differences in mRNA turnover may be caused by the involvement of additional *trans*-acting regulatory factors which bind directly to RNA or act via protein–protein interactions to other RNA-binding proteins. Proteins binding to URE likely interact with proteins binding to adjacent sequences, such as ARE, and thus overcome their destabilizing effects. It is also plausible that the binding of proteins to URE prevents, or hinders, the interaction between the AREs and destabilizing factors.

The identified proteins bound to mRNA within a region that contains regulatory sequences and were up-regulated by a stimulus that promotes GM-CSF expression. These observations suggest a function of these proteins in mRNA turnover.

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