Leukemia (1998) 12, 520–531

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Characterization of proteins binding the 3′ regulatory region of the IL-3 gene in IL-3-dependent and autocrine-transformed hematopoietic cells

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Previously we documented the prolongation of the IL-3 mRNA half-life in an autocrine-transformed cell line. This cell line has an intracisternal type A particle transposition in the IL-3 mRNA 3′ untranslated region which displaced four out of six AUUUA motifs involved in IL-3 mRNA destabilization. In this study, the proteins binding to the IL-3 mRNA AU-rich elements (ARE) were examined. Specific protein binding was detected to the wild-type IL-3 ARE region which contained 6 AUUUA motifs (AU_6). In contrast, no binding was detected to the mutated IL-3 ARE region which contained only two AUUUA motifs (AU_2). Proteins with apparent molecular weights of 36, 40, 43, 46, 55, 57, 68 and 95 kDa were bound to AU_6 motif. The hnRNP C and AUF-1 (hnRNP D) proteins were determined to be two of the IL-3 ARE binding proteins. Incubation of protein extracts with antibodies to hnRNP C and AUF-1 significantly decreased the protein binding to the IL-3 ARE. Treatment of IL-3 dependent cells with calcium ionophores eliminated the proteins binding to the ARE in wild-type IL-3-dependent FL5.12 cells and also resulted in the accumulation of IL-3 mRNA transcripts with a long half-life. These results indicated that there was a specific complex which bound the IL-3 mRNA 3′ ARE. Mutations which truncate the IL-3 ARE eliminate the ability of proteins to bind this regulatory region and can result in autocrine transformation due to the presence of IL-3 mRNA transcripts with a long half-life.
Keywords: IL-3; cytokines; mRNA stability; autocrine transformation; post-transcriptional regulation

Introduction

The proliferation of many hematopoietic precursor cells is promoted by interleukin-3 (IL-3), granulocyte/macrophage-colony stimulating factor (GM-CSF), stem cell factor (SCF) and other growth factors.[1–5] Removal or depletion of these growth factors from cytokine-dependent cells results in apoptosis, programmed cell death. Hence some of these cytokines, eg IL-3 and GM-CSF are also referred to as survival factors. Once 'normal' hematopoietic cells lose their growth factor dependency, they often become leukemic.[1–5]

IL-3 and GM-CSF exert their biological activity by binding to the IL-3 and GM-CSF receptors, respectively (IL-3R and GM-CSFR[6–11]). These receptors are comprised of a ligand-specific α-subunit and a common β-subunit (β_c), which is essential for signal transduction.[6–9] Abnormal expression of these receptors can contribute to leukemogenesis.[7–10] These receptors induce multiple signaling pathways which include oncoproteins, protein kinases and transcription factors.[11–13] If certain of these proteins are aberrantly regulated, a leukemia may arise.[11–13]

IL-3 expression is regulated by both transcriptional and post-transcriptional mechanisms. The 3′ flanking region of IL-3 gene contains an AU-rich element (ARE) including six ATTTA motifs, which confer instability to the IL-3 mRNA.[14,15] Calcium ionophores have been reported to stabilize IL-3 mRNA in some mast cells, indicating that calcium mobilization can lead to elongated IL-3 mRNA half-lives.[16] In contrast, immunosuppressive drugs such as cyclosporin A[17] and rapamycin[18] will destabilize IL-3 mRNA in certain autocrine-transformed tumors. These drugs may, in the future, be useful in the treatment of certain types of leukemia.

AREs are found in many short-lived proto-oncogene and cytokine mRNAs. These mRNAs contain an AU-rich motif of 30–80 bases within their 3′ untranslated region (UTR).[19,20] Based on sequence consensus and functional studies, Chen and Shyu[21–23] divided AREs into non-AUUUA and AUUUA-containing AREs. The non-AUUUA AREs have a long continuous U-rich region, eg c-*jun* ARE. Chen and Shyu further subdivided AUUUA-containing AREs into two classes: class I contains one to three copies of AUUUA motifs coupled with a long continuous U-rich sequence, eg c-*fos* ARE and AREs from some other transcription factors; class II contains multiple reiterations of an AUUU tetranucleotide, eg AREs from cytokine mRNAs, including IL-3 mRNA.[15] Site-directed mutagenesis studies performed with the granulocyte/macrophage colony-stimulating factor ARE indicated that three AUUUA motifs was the minimum for destabilizing mRNA.[24] Later investigations performed on IL-3 mRNA indicated that a cluster of three adjacent AUUUA motifs, either the 5′ three or the 3′ three, was able to destabilize IL-3 mRNA.[15] Recent experiments performed with c-*fos* and synthetic AREs have shown that the functional motif within the AREs was a UUAUUUA(U/A)(U/A) nonamer.[25,26]

Many investigators have demonstrated that there are proteins binding to ARE region that are involved in mRNA destabilization.[26–37] Brewer[31] and Zhang *et al*[32] purified and molecularly cloned an AUF1 factor that binds to the ARE region of c-*myc* and GM-CSF mRNA. An antibody specific for AUF1 cross-reacts with 37 and 40 kDa polypeptides which both bind to the ARE sequence. Furthermore, the αAUF1 antibody also recognizes a 45 kDa protein which is immunologically related to the two AUF1 proteins.[32] These AUF1 proteins are phosphorylated, localized in both the nucleus and the cytoplasm, and can be found in a complex.[32] The affinity of this protein for its substrates was negatively correlated with mRNA stability.[38] The involvement of AUF1 in the regulation of a specific mRNA's stability was also demonstrated.[39] Recently it was shown that AUF1 is the same as heterogenous nuclear ribonucleoprotein D (hnRNP D) and is also a component of the α-globin mRNA stability complex.[40]

Our previous work analyzed IL-3 mRNA stabilization in an autocrine-transformed IL-3-independent cell line, FL-*IL3*-R.[5,14,41,42] There is a rearranged IL-3 gene in this cell line. This rearrangement was due to the transposition of an intracisternal type A particle (IAP) into the 3′ UTR of the IL-3 gene.[14] The transposed IAP element displaced four out of six ATTTA motifs

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in the 3′ UTR of the IL-3 gene.[14] The IL-3 mRNA half-life was elongated ($t_{\frac{1}{2}} = 16 \pm 4$h) compared to wild-type IL-3 mRNA ($t_{\frac{1}{2}} = 0.5$–1 h).[5,14,39,40] The combination of transcriptional induction of IL-3 expression by the IAP long terminal repeat and mRNA stabilization due to disruption of the destabilizing sequence, resulted in abnormal IL-3 expression and autocrine transformation.[41,42] In the present study, the proteins which bind to IL-3 3′ UTR region and are involved in mRNA degradation control were characterized in IL-3-dependent and autocrine-transformed cells. The identification and partial characterization of proteins which bind the IL-3 mRNA region in IL-3-dependent and autocrine-transformed cells has not been described previously. Moreover, this work also documents that calcium ionophores can eliminate the binding of proteins to the IL-3 ARE and prolong IL-3 mRNA stability in IL-3-dependent cells. Thus, these studies further our understanding of how the deletion of the IL-3 ARE can result in autocrine transformation of hematopoietic cells.

Materials and methods

Cell lines and cytoplasmic protein extraction

The IL-3 dependent FL5.12[43] cells were maintained in a humidified 5% CO_2 incubator with Dulbecco's modified Eagle's medium (DMEM), containing 50 μM 2-mercaptoethanol (Sigma, St Louis, MO, USA) and 5% heat-inactivated iron-supplemented defined bovine calf serum ((CS), Hyclone, Logan, UT, USA) and supplemented with 20% clarified WEHI-3B (D^-) conditioned medium as a source of IL-3. The human cytokine-dependent TF-1 cells[44] were grown in DMEM + 10% FBS (Hyclone) containing 1 ng/ml recombinant human granulocyte/macrophage colony-stimulating factor (GM-CSF, R&D Systems, Minneapolis, MN, USA). Factor-independent lines FL-*IL3*-R1,[45] EL-4[46] and K562[47] cells were cultured in DMEM + CS in the absence of exogenous IL-3. In some experiments, the cells were treated with 50 nM phorbol 12-myristate 13-acetate (PMA; Sigma), 10 μM calcium ionophore (A23187; Boehringer Mannheim, Indianapolis, IN, USA), 5 μg/ml actinomycin D (Sigma) or 20 μg/ml cycloheximide (Sigma).

Cytosolic proteins were extracted according to the previously described procedure[33] with minor modifications. 5×10^7 cells were washed twice in cold PBS, which were then lysed in 1% Triton X-100 (Sigma), 10 mM PIPES (pH 6.8; Sigma), 100 mM KCl, 2.5 mM $MgCl_2$, 300 mM sucrose, 2 mM dithiothreitol (DTT; Sigma) and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma) by resuspension and incubation on ice for 10 min. The crude cell lysates were then centrifuged at 12 000 *g* for 10 min to pellet the nuclei and the supernatant was collected. Cytoplasmic extracts were stored at –80°C. The protein quantification was performed by using the Bradford reagent (Bio-Rad, Richmond, CA, USA).

RNA probes

The templates for the wild-type IL-3 3′ UTR probe containing six AUUUA motifs (AU_6) and the rearranged IL-3 3′ UTR probe containing two AUUUA motifs (AU_2) were constructed as described previously.[42] After being linearized by *Sma*I and *Hin*cII digestion and transcription driven by T7 RNA polymerase (Ambion, Austin, TX, USA), a 172-nucleotide and a 167-nucleotide riboprobe were obtained for wild-type and rearranged IL-3 3′ UTR, respectively. The sequence for AU_6 probe is: GGGCGAAUUU GCAACUCUCC UUUGGCUUUA CCUAAUUAUG UUCCUAUUUU AUUCCAUUAA GGCUAUUUAU UUAUGUAUUU AUGUAUUUAU UUAUUUAUUG CCUUCUGUGA UGUGAGUAUA UCUGUUUUAG CUGAGGAGGA GUUUCUCCAA AGAAAAUUCG AGCUCGGUAC CC. The sequence for AU_2 probe is: GGGCGAAUUC GAGCUCGCUU UACCUAAUUA UGUUCCUAUU UUAUUCCAUU AAGGCUAUUU AUUUAUGU*GU GGGAAGCCGC CCCCACAUUC GCCGUCACAA GAUGGCGCUG ACAUCCUGUG UUCUAAGUUG GUAAACAAAU AAUCUGCGCA UGAGCCAAGC UAGAGUC*. The AUUUA motifs are underlined. The sequence from the IAP is italicized in the AU_2 probe. A 180 nucleotide long, non-specific, non-AUUUA containing probe (AU_0) was obtained by cleavage of a 280 base pair long fragment from pGEM3Zf(–) by *Pvu*II, followed by an *in vitro* transcription reaction driven by SP6 RNA polymerase (Ambion). The *in vitro* transcription reactions were performed by incubating 1 μg of linearized DNA template in a 20 μl mixture containing transcription buffer, 0.5 mM of ATP, GTP, CTP, 50 μCi α-^{32}P UTP (3000 Ci/mmol, 10 mCi/ml; NEN, Boston, MA, USA), and 10 units of T7 RNA polymerase at 25°C for 1 h. Subsequently the template DNAs were removed by the addition of 2 units of RNase-free DNase I (Life Technologies, Gaithersburg, MD, USA), and incubated at 37°C for 30 min. The yield of the probe was approximately 1×10^8 c.p.m./μg. Poly(A), poly(G), poly(C), poly(U) and poly(I) oligonucleotides were in some cases used to compete the protein binding to the radiolabeled RNA probes and were purchased from Sigma and 25 ng of each oligonucleotide was added.

RNA electrophoretic mobility shift assay (EMSA)

Cytosolic proteins 2.5–30 μg were mixed with 5×10^4 to 1×10^5 c.p.m. gel purified RNA probe and incubated in a RNA protein binding buffer[48] (10 mM HEPES, pH 7.8, 5 mM $MgCl_2$, 50 mM KCl, 10% glycerol, 1 μg/ml yeast tRNA, 1 mM DTT; in a 25 μl total volume. The binding reactions were carried out at 30°C for 20 min. Then, 10 μg RNase A and 10 units of RNase T1 (Ambion) were added and incubated at 37°C for 10 min to digest the unbound RNAs. The samples were loaded on 8% non-denaturing polyacrylamide gels (acrylamide/bisacrylamide ratio 60:1), which were pre-electrophoresed for 30–60 min at 13 v/cm in 0.5 × TBE (1 × TBE = 0.089 M Tris, 0.089 M Borate and 0.002 M EDTA, final pH = 8).[49] The gels were electrophoresed at the same current until the xylene cyanol band of the tracking dye was approximately two inches from the end of the plates. The gels were subsequently dried and subjected to autoradiography. Proteinase K (Life Technologies) was added to some binding reactions at a final concentration of 2 mg/ml and incubated for 30 min at 37°C. In some experiments, either the αAUF1 antibody or preimmune serum was preincubated with cytoplasmic proteins for 15 min prior to the addition of radiolabeled riboprobes. The αAUF1 antibody was generously provided by Dr G Brewer (Wake Forest University, Winston-Salem, NC, USA).[32]

Some binding reaction mixtures were placed on ice and UV-crosslinked by using a Stratalinker 1800 (Stratagene, La Jolla, CA, USA) for 5 min at 3000 mW/cm^2.[33,49] Subsequently, the reactions were subjected to RNase A/T1 digestion and analyzed on 12% SDS-PAGE, followed by autoradiography.

Reverse transcriptase polymerase chain reaction (RT-PCR)

RNA isolation and RT-PCR were performed as described[41] with specific primers for IL-3 (upstream: 5′-AAT-CAGTGGCCGGGATACCC-3′ and downstream: 5′-CGAAAT-CATCCAGATCTCG-3′), and β_2-microglobulin (upstream: 5′-TTCTCTCACTGACCGGCCTG-3′ and downstream: 5′-CGAAATCATCCAGATCTCG-3′), defining a 200 bp and a 308 bp fragment cDNA fragment, respectively. Total RNA of 2 μg was used in the RT-PCR reactions. Cells were treated with actinomycin D as described.[19]

Western blot analysis

Cytoplasmic extracts of 40–80 μg were separated by SDS-PAGE (4% stacking phase and 12% resolution phase). Proteins were then electroblotted onto 0.2 μm polyvinylidene difluoride membranes (PVDF; Bio-Rad) with a Bio-Rad semi-dry transfer apparatus as described.[50] After blotting, the membrane was blocked in TBS-T buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.6) with 5% non-fat dried milk, for 1 h at room temperature, with shaking. Then the diluted primary antibody (1:5000-fold dilution for αAUF1, 1:1000-fold dilution for αhnRNP A1 and C) was added and incubation was carried out overnight at 4°C, with shaking. On the following day, the filter was washed three times for 5 min with TBS-T buffer, and incubated with the appropriate diluted secondary antibody (1:5000-fold dilutions, anti-rabbit IgG/horse radish peroxidase (HRP) for αAUF1 and αmouse IgG/HRP for αhnRNP A1 and C (Amersham, Arlington Heights, IL, USA)) for 1 h at room temperature, with shaking. The filter was then washed five additional times. The signals were detected by ECL Western blotting kit according to the manufacturer's protocol (Amersham) and visualized after exposure to Kodak film (Rochester, NY, USA).

Northwestern blot analysis

After electrophoresis of the proteins prepared from the appropriate cell lysates and transfer onto the PVDF membranes, the membrane-bound proteins were renatured in 10 mM HEPES (pH 7.8), 3 mM $MgCl_2$, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.2% Nonidet-P40, 5% glycerol and 5 mg/ml BSA overnight at room temperature. The next day, the membranes were transferred to RNA–protein binding buffer and incubated with specific radio-labeled probes at 1×10^5 c.p.m./ml for 2 h at room temperature, followed by three washes in RNA–protein binding buffer. The first two washes were carried out at room temperature and the last wash was at 37°C and included 25 mg/ml RNase A (Sigma). The signals were visualized after exposing the dried membranes to PhosphorImager screens followed by analysis with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

Immunoprecipitation of ARE-binding proteins

After the cytoplasmic lysates were incubated with the radiolabeled AU_6 probe, UV-crosslinked, and digested with RNase A/T1, a 1:500-fold dilution of respective monoclonal antibodies, (mouse 4F4 (specific for hnRNP C), 9H10 (specific for hnRNP A1)), a polyclonal rabbit αAUF1 antiserum, or a pre-immune antiserum were added to the RNA-protein mixture. The mixtures were incubated at 4°C overnight with 40 μl of protein A-sepharose beads (Pharmacia) for polyclonal antibodies, or 10 μl protein G-agarose beads (Life Technologies) for monoclonal antibodies in a buffer consisting of 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.5% Triton X-100 and 1 mM PMSF. The protein G-agarose beads were pre-incubated with 10 μl rabbit αmouse IgG Fc (Accurate Chemical & Scientific Corporation, Westbury, NY, USA) for 1 h before use. The next day, the beads were pelleted after a brief centrifugation at 12 000 *g*, washed five times in PBS, boiled in Laemmli sample buffer,[32–34] analyzed by 12% SDS-PAGE, and followed by autoradiography. The mouse 4F4 and 9H10 ascites derived monoclonal antibodies were provided by Dr GS Dreyfuss (University of Pennsylvania, Philadelphia, PA, USA).[51–53]

Results

Proteins bind specifically to the AU_6 probe, but not the AU_2 or AU_0 probes

Since there was a 20-fold difference in IL-3 mRNA stability observed between cells containing the wild-type (AU_6) or the mutated (AU_2) 3′ UTRs,[5,41,42] we proposed that there might be a difference in protein binding to the wild-type and mutated IL-3 mRNA 3′ UTRs. RNA protein binding experiments indicated that there was protein binding detected to the wild-type IL-3 mRNA 3′ UTR probe (Figure 1a); whereas no protein binding was observed to either the rearranged AU_2 probe, or a non-specific, non-ARE containing AU_0 probe (Figure 1a). Proteinase K was included in some binding reactions, which abrogated the binding to the wild-type AU_6 probe (Figure 1b). This indicated that the observed bands were the result of the interaction of proteins with the labeled RNA. A concentration effect of cytoplasmic protein on riboprobe binding was determined by incubating different amounts of cytoplasmic extracts in the binding assay. These results indicated that the binding of proteins to the wild-type probe was proportional to the amount of protein added (Figure 1c). Similar results were observed with cytoplasmic extracts prepared from both FL5.12 (Figure 1) and autocrine-transformed FL-*IL3*-R cells (data not presented).

To further analyze the proteins in the binding complex, the UV-crosslinking and label transfer technique was applied.[33,49] After the binding reactions, the protein–RNA mixtures were exposed to UV light, and the proteins were separated by 12% SDS-PAGE. Proteins with apparent molecular weights of 36, 40, 43, 46, 55, 57, 68 and 95 kDa were bound to the AU_6 probe, whereas barely any protein binding was observed with the AU_2 probe. Similar protein:RNA binding patterns were observed in both FL5.12 and FL-*IL3*-R cell lines (Figure 2a). The proteins binding to AU_6 probe were competed away by poly(U), but not by poly(A), poly(G), poly(C) or poly(I) (Figure 2b), which indicated that the proteins binding to IL-3 3′ UTR ARE region had high affinity to a U-rich sequence. Furthermore, the proteins binding to wild-type probe were competed away by excess amounts of non-radiolabeled-specific probes, but not by excess amounts of non-radiolabeled non-specifc probes (Figure 2c).

To further characterize the binding activity, Northwestern blot analysis was performed with AU_6, AU_2 and AU_0 probes. Cytoplasmic proteins were electrophoresed on SDS polyacrylamide gels, transferred and immobilized onto nitrocellulose

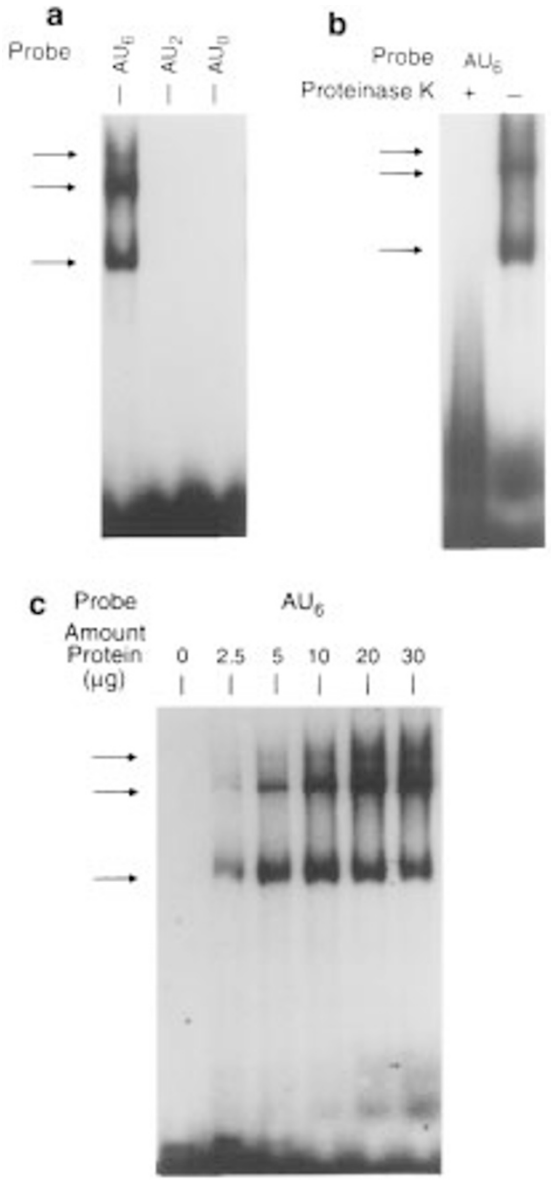

Figure 1 Proteins bind to the AU_6 ARE probe but not to either the AU_2 or AU_0 ARE probes. Cytoplasmic extracts from FL5.12 cells were incubated with the radiolabeled wild-type IL-3 ARE probe (AU_6 172 nt long), the mutated IL-3 ARE probe (AU_2, 167 nt long), or a non-specific probe (AU_0, 182 nt long) which did not contain any AUUUA motif. These mixtures were digested with RNase A/T1 and resolved by electrophoresis on a native polyacrylamide gel. (a) Protein:RNA binding was detected with the wild-type IL-3 ARE probe (AU_6), but not with the mutated probe (AU_2), or the non-specific probe (AU_0). (b) Treatment with proteinase K abolished the protein binding to wild-type IL-3 ARE probe (AU_6). (c) Concentration effect of protein binding to the wild-type IL-3 ARE Probe (AU_6). 0–30 μg of cytoplasmic proteins were used in the binding reactions. The protein:RNA binding was proportional to the amount of the protein added in the binding reactions.

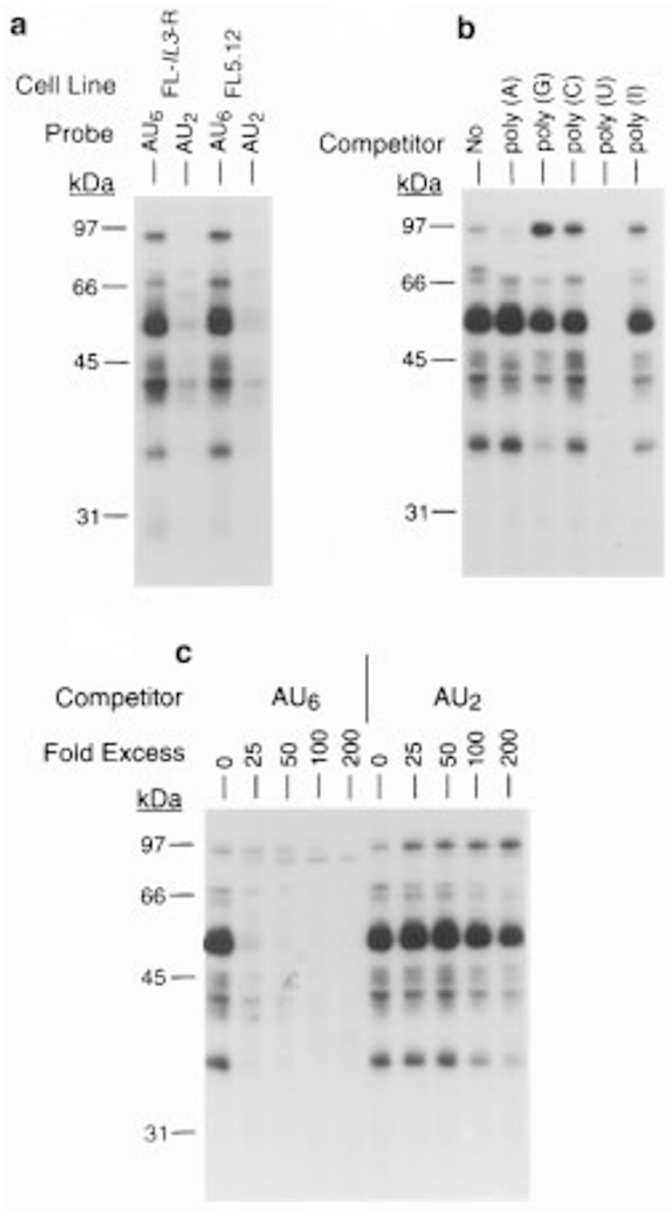

Figure 2 Distinct protein binding to the AU_6 ARE probe. After the binding reactions, the protein:RNA mixtures were exposed to UV light as described in Materials and methods. They were then subjected to RNase A/T1 digestion and resolved by electrophoresis on 12% SDS polyacrylamide gels. (a) The binding reactions were performed with either the wild-type IL-3 ARE probe (AU_6), or the mutated IL-3 ARE probe (AU_2) with cytoplasmic extracts isolated from either the autocrine-transformed FL-*IL3*-R or the IL-3-dependent FL5.12 cells. (b) The ability of the protein-binding to AU_6 probe to be competed away with non-radiolabeled poly(A), poly(G), poly(C), poly(U) or poly(I). 25 ng of each polynucleotide was added. Only the poly(U) competed away the binding. (c) The ability of protein-binding to the AU_6 probe to be competed away with an excess amount of non-radiolabeled AU_6 or AU_2 probes was examined. Only the AU_6 probe competed away the binding.

filters and subsequently incubated with radiolabeled probes. Consistent with previous observations, protein:RNA binding was detected with the AU_6 probe, while no binding was observed with the AU_2 or AU_0 probes (Figure 3). With the AU_6 probe, a band of 36 kDa was detected in two murine cell lines (FL5.12 and the T cell thymoma EL-4), and a 95 kDa protein was detected in FL5.12 cells (Figure 3). In the human hematopoietic cell lines examined (TF-1 and K562), proteins with molecular weight 43, 68, 95 and 105 kDa were detected by Northwestern blot analysis to bind the murine IL-3 mRNA ARE probe (AU_6) (Figure 3). As with murine cells, there was no protein:RNA binding observed with the AU_2 or AU_0 probe in cell extracts prepared from human cells. This indicated that

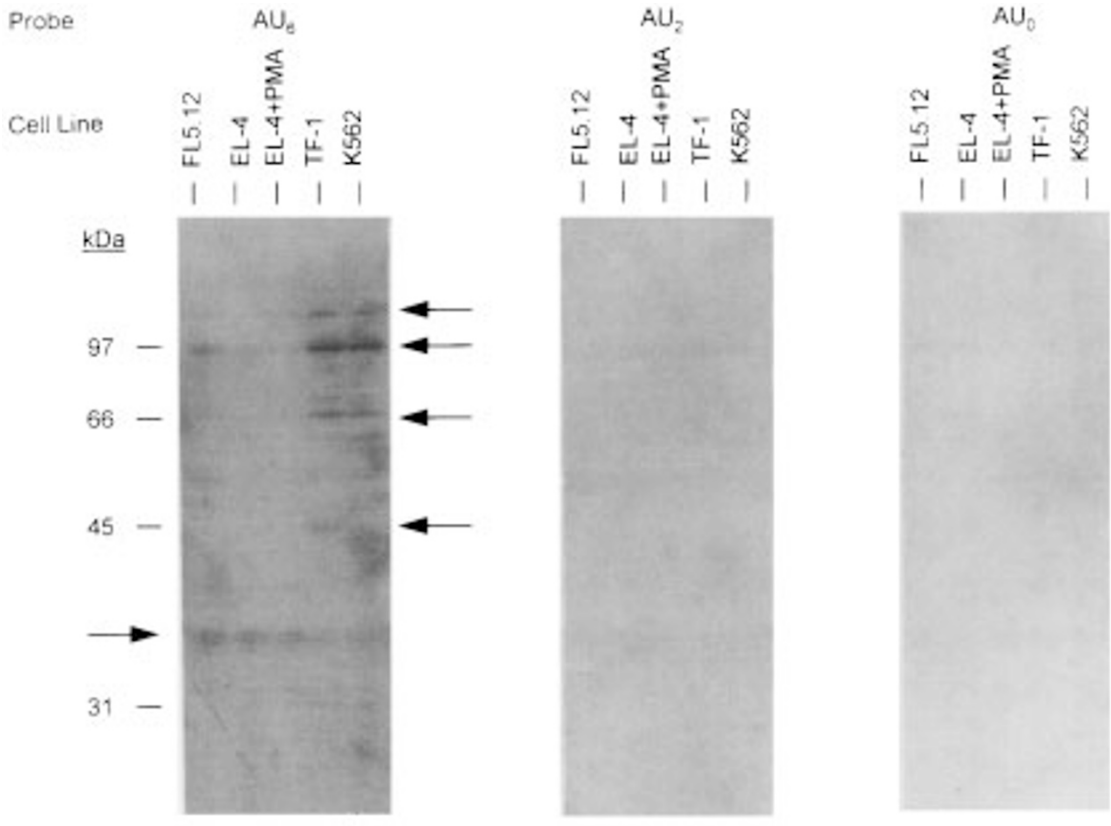

Figure 3 RNA–protein binding detected by Northwestern blot analysis. Cytolplasmic proteins were separated by 12% SDS-PAGE before being transferred to nitrocellulose membranes. The membranes were then incubated with radiolabeled AU_6, AU_2 or AU_0 probes at a concentration of 1×10^5 c.p.m./ml. The cytoplasmic extracts were isolated from two mouse cell lines, FL5.12 and EL-4 (a T cell thymoma, which was grown either normally or exposed to PMA for 4 h), and two human erythroid lines, TF-1 and K562. Arrows indicate the radio-labeled protein bands.

some proteins which bound the murine IL-3 ARE might be similar in murine and human hematopoietic cells (95 kDa), while others appeared to have different molecular weights. In summary, Northwestern analysis indicated that murine proteins of 36 and 95 kDa bound the murine RNA under the conditions of these experiments.

AUF1 (hnRNP D) and hnRNP C are included in the IL-3 mRNA 3′ ARE protein binding complex

AUF1 (hnRNP D) proteins and certain other hnRNPs have been shown to bind to specific mRNA ARE regions and may be involved in mRNA stability control.[31–34] Whether they are also involved in IL-3 mRNA ARE binding is unknown. The expression of the AUF1 protein in the cell lines used in this study was examined by Western blot analysis. The human αAUF1 antibody cross-reacted with murine proteins (Figure 4a). Three bands were detected with the αAUF1 antibody with molecular weights of 40, 43 and 46 kDa. These are the three immunologically related isoforms of the AUF1 protein.[32] There were nearly equal levels of AUF1 proteins detected in K562, TF-1 and FL5.12 cells. Slightly lower levels of AUF1 proteins were detected in EL-4 and EL-4 cells treated with PMA (Figure 4a). The AUF1 protein was also detected in the autocrine-transformed FL-*IL3*-R cells and similar levels were observed as in the FL5.12 cells (data not presented).

To determine whether the AUF1 protein was included in the IL-3 mRNA 3′ UTR binding complex, the αAUF1 antibody was pre-incubated with cytoplasmic proteins in some binding reactions. Pre-treatment of cytoplasmic proteins with the αAUF1 antibody decreased the protein binding to the AU_6 probe by two- to three-fold, while the preimmune serum did not affect the binding (Figure 4b and c). This result demonstrated that the AUF1 protein was likely contained in the binding complex. Moreover, since the αAUF1 antibody decreased the two bands on a native gel, it is conceivable that these bands included AUF1 proteins.

The specificity of this antibody interaction was further examined by dose–response experiments with this and additional antibodies (Figure 5). Increasing the concentration of the αAUF1 antibody decreased the amount of RNA:protein complex observed (Figure 5). Certain other hnRNPs have been demonstrated to bind to ARE regions.[33,34,51,52] Moreover, some of these hnRNPs have similar molecular weights as certain of the IL-3 ARE-binding proteins, such as hnRNP A1 (which has a molecular weight of 36 kDa) and hnRNP C (which has molecular weights of 41 and 43 kDa).[33,34,51,52] The same dose–reponse experiments were performed with the 9H10 antibody which recognizes the αhnRNP A1 protein and it did not decrease the RNA:protein binding (Figure 5) indicating the specificity of these experiments. Finally, these experiments were also performed with the 4F4 antibody which recognizes the hnRNP C protein. The antibody to this protein also decreased the level of RNA:protein complex observed at the higher concentrations. Thus the ability of the αAUF1 and hnRNP C antibodies to decrease the RNA:protein complex was specific.

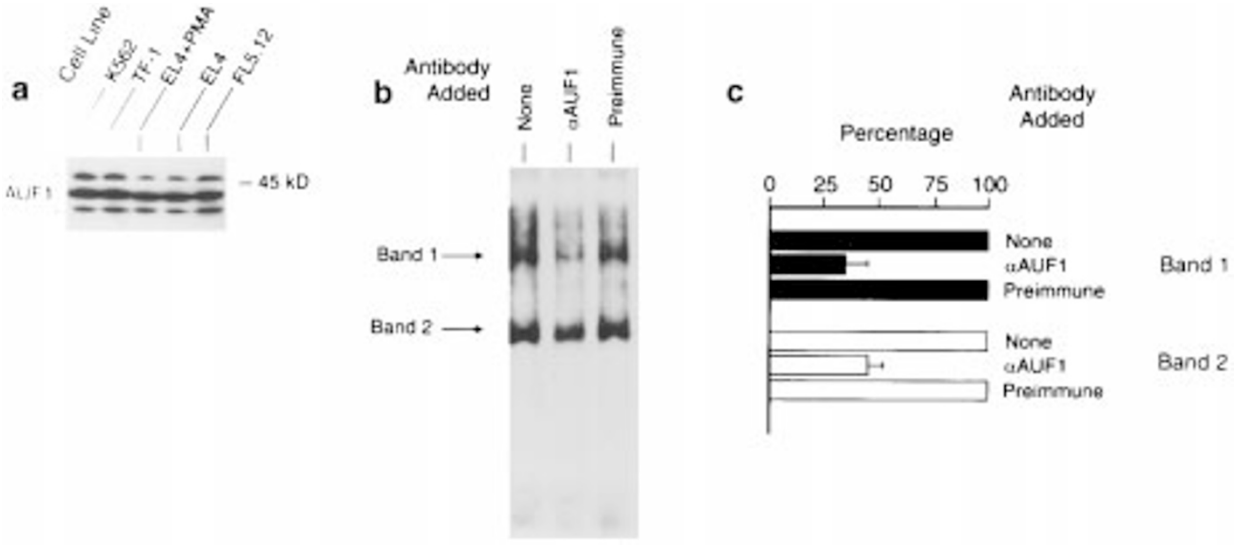

Figure 4 Presence of AUF-1 (hnRNP D) in the IL-3-ARE binding complex. (a) Detection of AUF1 expression by Western blot analysis. Cytoplasmic proteins were isolated from two human cell lines, K562, TF-1, and two mouse cell lines, EL-4 (with or without PMA treatment) and FL5.12 cells. The proteins were resolved by 12% SDS-PAGE before being transferred to a nitocellulose membrane. The membrane was then immunoblotted with an αAUF1 antibody. (b) Cytoplasmic proteins from FL5.12 cells were pre-incubated with either an αAUF1 antibody or a preimmune serum before adding the radiolabeled AU_6 probe. The radiolabeled probe was then added, and the reaction mixtures were digested with RNAse A/T1 and resolved by native polyacrylamide gel electrophoresis. (c) Quantification of the protein–RNA binding inhibited by an αAUF1 antibody. This experiment was repeated three times with similar results.

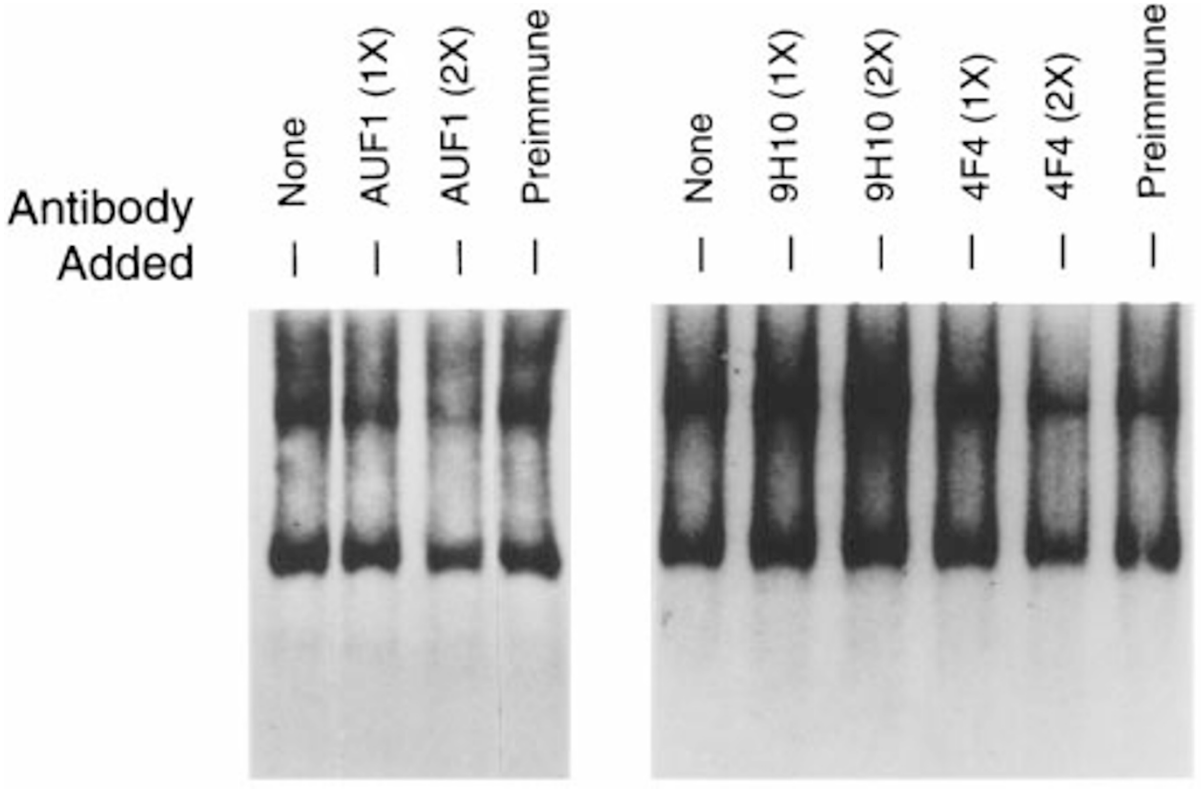

Figure 5 Protein binding to the wild-type IL-3 ARE probe was blocked in a dose-dependent fashion by antibodies to AUF-1 and hnRNP C. Cytoplasmic proteins from FL5.12 cells were pre-incubated with different doses of the following antibodies, αAUF-1, 9H10 (αhnRNP A1) and 4F4 (αhnRNP C) or a preimmune serum before adding the radiolabeled AU_6 probe. The radiolabeled probe was then added, and the reaction mixtures were digested with RNase A/T1 and resolved by native polyacrylamide gel electrophoresis. $1\times = 10\ \mu g/ml$, $2\times = 20\ \mu g/ml$.

To further confirm that AUF1 and hnRNP C were involved in the binding to the IL-3 ARE region, immunoprecipitation experiments were performed on UV-crosslinked protein:RNA complexes. After UV-crosslinking the protein:RNA complex, the reaction mixture was immunoprecipitated with the different antibodies. The precipitated proteins were resolved on SDS polyacrylamide gel and visualized by autoradiography. Only the RNA binding proteins which were recognized by the specific antibodies were visible on the autoradiograph. The αAUF1 antibody immunoprecipitated three proteins which bound the IL-3 ARE probe (Figure 6, lane 3), and displayed similar molecular weight as detected by Western blot analysis using an αAUF1 specific antibody (Figure 4a). In contrast, the pre-immune antisera did not recognize the proteins which bound the IL-3 ARE probe (Figure 6a, lanes 2 and 7).

To determine if hnRNP A1 and C were among the proteins that bound to IL-3 ARE region, monoclonal antibodies specific for hnRNP A1 (9H10) and hnRNP C (4F4) were used to immunoprecipitate the cytoplasmic proteins which bound to the radiolabeled IL-3 ARE probe. As shown in Figure 6a, immunoprecipitation with a monoclonal antibody against hnRNP C (4F4), gave rise to two radiolabeled bands which were 41 and 39 kDa (lane 6). This result indicated that hnRNP C proteins were included in the binding complex.

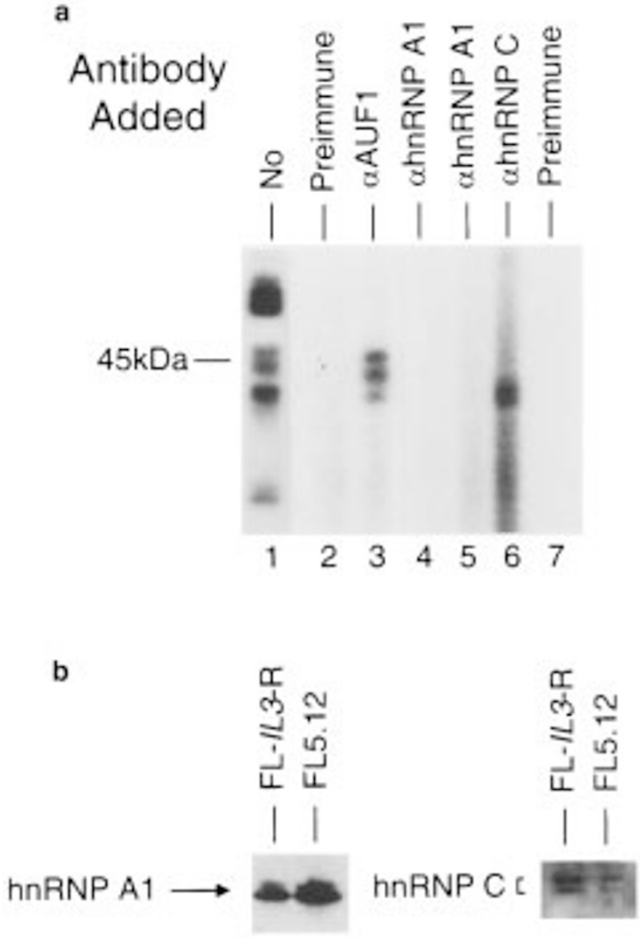

Figure 6 Immunoprecipitation of IL-3 ARE-binding proteins with antibodies specific for AUF1, hnRNP C and A1 proteins. (a) Cytoplasmic proteins were incubated with radiolabeled AU_6 probe, UV-crosslinked, and digested with RNase A/T1. The binding mixtures were subsequently immunoprecipitated with preimmune antiserum (Lanes 2 and 7); an αAUF1 specific antibody (lane 3); αhnRNP A1 monoclonal antibody (9H10, lanes 4 and 5), or an α-hnRNP C monoclonal antibody (4F4, lane 6). The precipitated proteins were resolved on 12% SDS polyacrylamide gel. Lane 1, protein:RNA binding following UV-crosslinking was resolved on SDS polyacrylamide gel. All the cytoplasmic proteins were isolated from untreated FL5.12 cells, except for lane 4, where the cytoplasmic proteins were extracted from FL5.12 cells that had been pre-treated with actinomycin D for 30 min. (b) The expression of hnRNP A1 and hnRNP C proteins was determined by Western blot analysis.

To determine whether hnRNP A1 was included in the protein:RNA binding complex, the UV-crosslinked complex was immunoprecipitated with the monoclonal αhnRNP A1 antibody, 9H10. This specific antibody did not precipitate the 36 kDa binding protein (Figure 6, lanes 4 and 5), even when the cells were pre-treated with actinomycin D, which induces the binding of a 36 kDa protein to IL-3 ARE region (data not presented). This result indicated hnRNP A1 was not involved in the binding to IL-3 ARE region. Western blot analysis was performed to detect the expression of hnRNP A1 and C proteins as well as to determine the effectiveness of these antibodies to recognize these proteins. The results in Figure 6b indicated these proteins were expressed in both FL5.12 and FL-*IL3*-R cells.

Overall, these results indicated that the AUF1 (hnRNP D) and hnRNP C proteins bind to the IL-3 mRNA ARE region and may play a role in IL-3 mRNA stability regulation. Moreover, these proteins are present in both IL-3-dependent and autocrine-transformed cells. This indicates that it is not their absence that results in the deregulation of mRNA stability in the autocrine-transformed FL-*IL3*-R cells, but rather their ability to bind the rearranged IL-3 ARE region.

Effects of calcium ionophores on protein binding to the ARE and IL-3 mRNA stability

Certain reagents have been documented to affect IL-3 mRNA accumulation.[16–18] Calcium ionophores stabilize IL-3 mRNA in some mast cells.[16,54] Phorbol esters can stimulate IL-3 mRNA expression in certain T lymphoma lines, such as EL-4 cells.[55,56] The effects of these drugs on the protein binding to the IL-3 ARE were determined. Treatment of FL5.12 cells with PMA did not considerably change the binding pattern (Figure 7a). In contrast, treatment of the cells with the calcium ionophore A23187 with or without PMA abolished the protein:RNA binding (Figure 7a).

RNAs were also isolated from these cells and the presence of IL-3 mRNA transcripts was examined by RT-PCR analyses. No IL-3 cDNAs were detected in either unstimulated or phorbol ester-treated FL5.12 cells. However, treatment of FL5.12 cells with the calcium ionophore A23187 induced IL-3 expression and the combined treatment with PMA and calcium ionophore resulted in a slightly higher level of IL-3 expression (Figure 7b). In both of these conditions, no proteins binding to the AU_6 ARE probe were detected. Thus, calcium ionophores altered protein binding to the IL-3 ARE and resulted in the accumulation of IL-3 mRNA transcripts.

To determine whether the calcium ionophore and PMA treatment prolonged the stability of IL-3 mRNA transcripts in FL5.12 cells, the half-life of IL-3 mRNA was determined. Culture of FL5.12 cells with PMA did not result in the detection of IL-3 mRNA transcripts (Figure 8). This dose of PMA was sufficient to induce the accumulation of IL-3 mRNA transcripts in the EL-4 T cell thymoma line.[55,56] Calcium ionophore treatment resulted in the detection of a low level of IL-3 transcripts with a prolonged (>4 h) half-life (Figure 7). The bands enco-

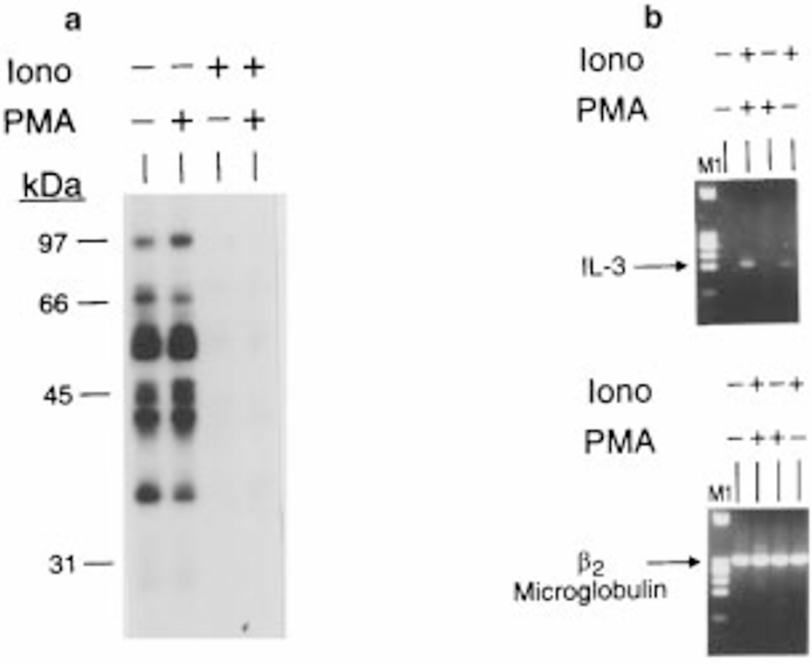

Figure 7 RNA–protein binding is altered after calcium mobilization. Cytoplasmic extracts were prepared from untreated FL5.12 cells, or cells treated for 4 h with PMA (50 nM), calcium ionophore (Iono, 10 mM), or PMA plus calcium ionophore. The cytoplasmic proteins were incubated with the radiolabeled probe (AU_6), UV-crosslinked, RNase A/T1 digested, and resolved by 12% SDS-PAGE (a). mRNAs were also isolated from the FL5.12 cells with the same respective treatments and IL-3 mRNA expression was detected by RT-PCR (b).

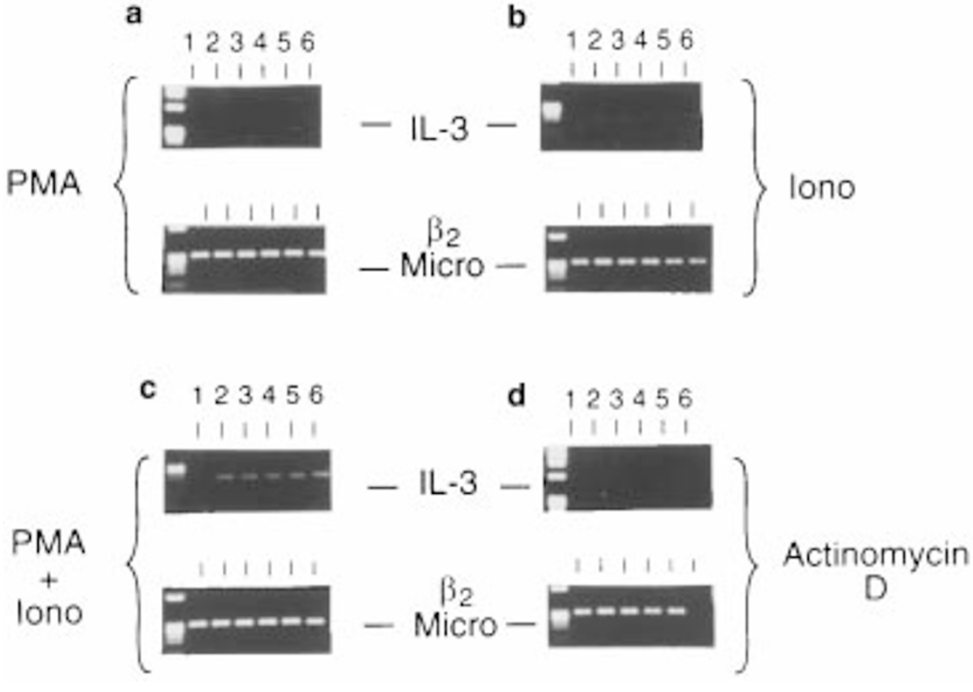

Figure 8 Calcium mobilization prolongs IL-3 mRNA stability in FL5.12 cells. FL5.12 cells were treated with calcium inophore (10 μM) or PMA (50 nM) for 4 h. Actinomycin D (5 μg/ml) was then added for increasing time periods. RT-PCR was then performed with 2 μg of FL5.12 RNA per sample. Lower amounts of FL5.12 mRNA did not result in the detection of IL-3 cDNAs. Lane 1, no stimulation; lane 2, treatment with either PMA, calcium ionophore or both for 4 h; lanes 3–6, the induced cells were subsequently treated with actinomycin D for: (3) 30 min (4) 1 h, (5) 2 h and (6) 4 h.

ding IL-3 cDNAs are more visible on the original photograph. The addition of both calcium ionophore and PMA resulted in a higher level of IL-3 cDNAs detected. As a control for the effects of actinomycin D on IL-3 mRNA accumulation, untreated cells were also cultured with actinomycin D. Actinomycin D by itself did not induce the accumulation of IL-3 mRNA transcripts in FL5.12 cells. In summary, the calcium ionophore treatment resulted in the accumulation of IL-3 mRNA transcripts with a prolonged half-life.

To determine whether the calcium ionophore treatment altered the levels of the AUF1 and hnRNP C proteins, protein extracts were prepared from these cells and Western blot analysis was performed. Calcium ionophore treatment did not reduce the levels of the AUF1 and hnRNP C proteins suggesting that the mechanism by which calcium ionophores prolonged the stability of IL-3 mRNA occurred independently of the level of these proteins (data not presented).

Discussion

Our previous work documented that the interruption of the six ATTTA motifs of the IL-3 gene by an IAP transposition stabilized IL-3 mRNA. Moreover, the IAP long terminal repeat stimulated IL-3 transcription. Together, these two events led to sufficient IL-3 synthesis to allow the cells to become transformed by an autocrine mechanism and form tumors in immunocompromised mice.[41,42] In this communication, we have further characterized the mechanism by which this mutation conferred IL-3 mRNA stabilization. Specific protein binding to the wild-type IL-3 mRNA ARE region was detected by native gel, UV-crosslinking and Northwestern blot analysis. All these approaches demonstrated that there was considerable protein binding to wild-type IL-3 ARE probe which contained six AUUUA motifs, whereas no binding was detected to the rearranged IL-3 ARE probe, which possessed two AUUUA motifs, or a non-specific probe which did not contain any AUUUA motifs.

Regulation of gene expression occurs by both transcriptional and post-transcriptional mechanisms. AREs are involved in the post-transcriptional control of mRNA stability.[57] However, the mechanisms by which they regulate mRNA destabilization are not clear. No evidence has indicated that AREs serve as endoribonucleolytic sites for enzymatic cleavage, yet this possibility has not been excluded. However, there have been studies which indicate that the presence of an ARE coincides with the rapid shortening of the poly A tail.[55–62] AREs may exert their effects by affecting mRNA deadenylation which is the first step in mRNA degradation.[57] There is growing evidence demonstrating that there are specific proteins which bind to the ARE region.[27–40] An important question which remains is how do these proteins function to regulate mRNA stability? Also, do the *cis* and *trans* elements work together to modulate mRNA stability? To answer these questions, an important first step is to characterize the proteins which bind to these regulatory regions.

Our RNA UV-crosslinking of RNA–protein binding assays indicated that there is more than one protein which binds to the IL-3 ARE region. Proteins with molecular weights of 36, 40, 43, 46, 55, 57, 68 and 95 kDa were determined to bind the wild-type IL-3 ARE. Thus, there can be quite a few proteins which interact with cytokine AREs. These ARE binding proteins are physiologically relevant since the prevention of this binding to the ARE is associated with prolonged cytokine mRNA half-life and tumorigenicity.[34,41,42]

The AUF1 (hnRNP D) protein was involved in the binding, as it was detected by RNA gel shift assay as well as immunoprecipitation of the binding proteins with a specific αAUF1 antibody. hnRNP C proteins were also determined to bind the IL-3 mRNA ARE region. Initially it was thought that the 36 kDa protein might be hnRNP A1. This was based on the properties of hnRNP A1 to bind mRNA and the binding to be induced by a transcriptional inhibitor, but not affected by a translational inhibitor. However, immunoprecipitation experiments did not support this hypothesis. Thus, while hnRNP A1 is expressed in these cells, it does not appear to bind the IL-3 3′ ARE.

Our results suggest that the formation of the protein complex is required for the IL-3 mRNA destabilization. When the AUUUA motifs were disrupted as occurred by the IAP transposition in the autocrine-transformed Fl-*IL3*-R cells, no protein binding to rIL3 ARE region was detected and the IL-3 mRNA was stable. FL5.12 cells did not normally express sufficient IL-3 mRNA transcripts to be detected by RT-PCR. When these cells were treated with calcium ionophore the formation of protein complex was abrogated. Calcium ionophores also stabilized IL-3 mRNA in these cells. Thus, elimination of this ARE binding complex was associated with increased IL-3 mRNA stability in FL5.12 cells. In contrast, treatment of FL5.12 cells with PMA did not appear to have any effects on protein binding or on IL-3 mRNA stabilization although it did synergize with the calcium ionophore treatment and resulted in the accumulation of higher levels of IL-3 mRNA transcripts.

IL-3 mRNA is also regulated by transcriptional mechanisms. The transcription factor NF-AT binds to certain sequences in the IL-3 promoter region.[4] Calcineurin is an important phosphatase which dephosphorylates certain proteins including NF-AT.[4,63] The dephosphorylated NF-AT enters the nucleus and activates cytokine gene expression, including IL-2 and IL-3.[4,63] Protein phosphorylation also plays a role in IL-3 mRNA stability control.[64–66] Clearly, there are other mechanisms besides the binding of proteins to the ARE which can control mRNA stability. Recent studies by Moroni and colleagues[17,18,64,67,68] have indicated that there are *trans*-acting factors which can regulate IL-3 mRNA stability in certain autocrine-transformed cells.

Disruption of the IL-3 ARE in FL-*IL3*-R cells resulted in the elimination of the protein binding to this regulatory region. A summary of these observations is presented in Figure 9. The IL-3 mRNA synthesized in FL-*IL3*-R cells did not bind the ARE binding proteins and the IL-3 mRNA remained stable and the cells were malignantly transformed. In contrast, the parental FL5.12 cells were IL-3-dependent, did not synthesize IL-3 mRNA transcripts and were not tumorigenic upon injection into nude mice.[5,41,42,45] Moreover, if any IL-3 mRNA was synthesized by FL5.12 cells, it would not be stable as there would not be any proteins which bound the IL-3 ARE. These and previous studies[41,42] document the importance of the regulation of cytokine ARE regions and indicate how the disruption of these sequences can result in malignant transformation.

Acknowledgements

This work was supported in part by a grant from the National Institutes of Health to JA McCubrey (R01 CA51025). We thank Dr PH Pekala for his advice on these experiments and the reading of the manuscript. We also thank Dr G Brewer and Dr GS Dreyfuss for providing us with the antibodies against the RNA binding proteins.

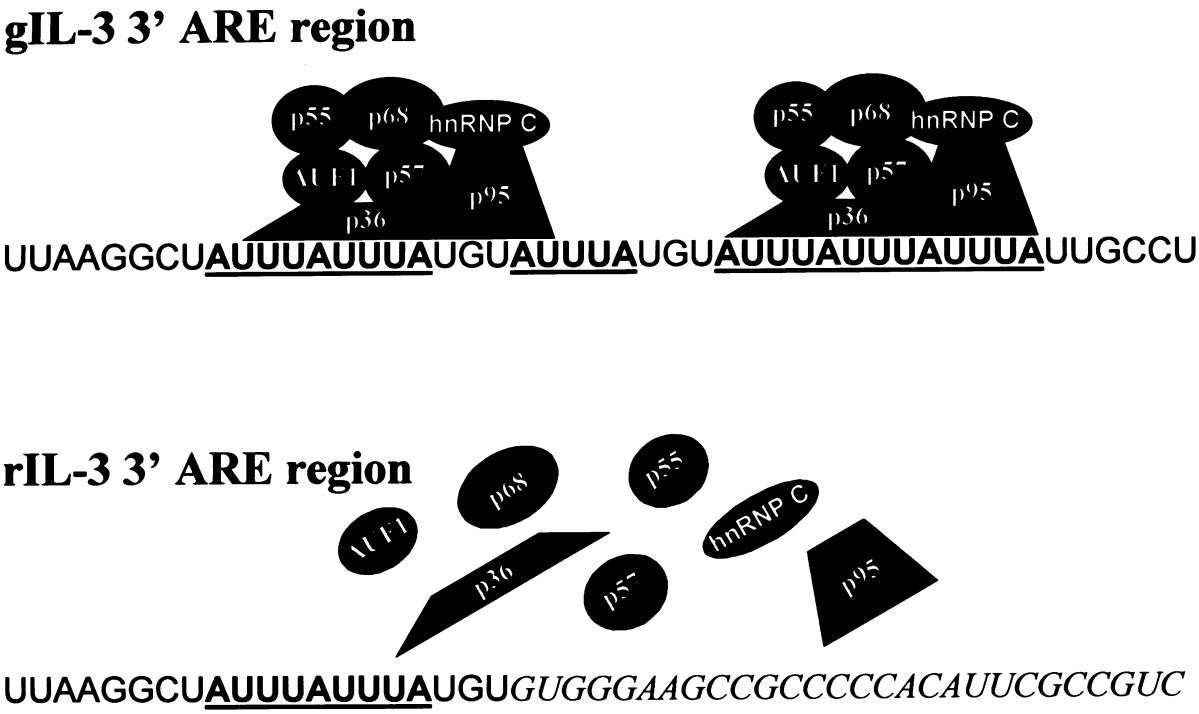

Figure 9 Diagram of protein binding to the IL-3 ARE in cells containing either the gIL-3 or the rIL-3 genes. Part of the sequences related to IL-3 mRNA stability region are indicated. AUUUA motifs are in bold and underlined, the sequences from the transposed IAP are italicized. The molecular weights of the RNA binding proteins are indicated. All of the proteins bind the gIL3 ARE, however, the two proteins detected by Northwestern analysis (p36 and p95) are shown in this two-dimensional model to have the most binding to the gIL3 ARE.

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