Post-transcriptional control of lymphokine gene-expression: role of mRNA-protein interactions

Ph. D. THESIS

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To My Father, who taught me how to enjoy life and how to be content...

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PREFACE

I became interested in post-transcriptional regulation of gene-expression while completing my post-doctoral training at Dartmouth Medical School. Our own investigation on the molecular background of activation-induced lymphokine production by T lymphocytes as well as work from other laboratories quickly turned our attention to post-transcriptional mechanisms as possible contributory events in lymphokine gene-expression. During the three years what I spent on this project, it became more and more obvious that mRNAs in the cell are being "processed" in an extremely intricate way. Following their nucleo-cytoplasmic passage, different mRNAs take different routes in their further metabolism in the cytoplasm. Some mRNAs are being stored "silent" for a relatively long time, while others get translated immediately. Various mRNAs differ considerably in their cytoplasmic turnover, falling thereby into two main classes. One class of messages possess long cytoplasmic half life, while mRNAs of the other class decay rapidly. The regulated mechanisms that contribute to the cytoplasmic fate of different mRNAs are termed post-transcriptional and they cover events from mRNA turnover and decay to mRNA sorting and translation. The functional role of RNA-protein interactions is well established in these processes. Specific sequence determinants or stem-loop structures at various portions of the mRNA as well as proteins capable of interacting with them have been implicated in a variety of systems. Moreover, it has been demonstrated that normally, translation (and possibly decay) of various mRNA species occurs at specific cytoplasmic locations, suggesting spatial regulation in their processing. Lymphokine gene-expression appears to be a valuable model in studying many components of post-transcriptional mechanisms. Uncovering key elements in this system

may lead to a better understanding of how cytoplasmic metabolism of mRNAs contribute to appropriate cell growth and differentiation.

This thesis is assigned to demonstrate the results of the work I have performed at Dartmouth and at the University Medical School of Pécs, aiming to gain a more expanded view on the various aspects of post-transcriptional means of lymphokine gene-expression. Although, I was involved in various projects that led to many intriguing observations relevant to the raised problems, yet, here, I focus only on the work that I conducted from experimental design to final interpretation. The Thesis also provides the opportunity to discuss the significance of the observations in the light of other experimental evidences and to set future directions along which additional work should be conducted.

Pécs, September, 1996.

ABBREVIATIONS

mRNA (messenger RNA)

**3`-UTR** (3` untranslated region)

ARE (AU-rich=adenin-uracyl rich instability sequence element)

eIF (eukaryotic initiation factor)

AUBP (AU-rich sequence element binding protein)

IL-2 (Interleukin-2)

γ-IFN (γ-Interferon)

TNF-α (Tumor necrosis factor-α)

PBMC (peripheral blood mononuclear cell)

hnRNP (heterogeneous nuclear ribonucleoprotein)

Act D (Actynomycin D)

ATCC (American Type Culture Collection)

dsDNA (double stranded DNA)

ssDNA (single stranded DNA)

DRB (5,6-dichloro-1-β-ribouranosylbenzimidazole)

ORF (open reading frame)

SDS-PAGE (sodium-dodecyl-sulphate polyacrylamide gel electrophoresis)

(Additional abbreviated names are listed in the presented publications).

Chapter 1: INTRODUCTION

The regulation of gene-expression is a complex process in all living organisms. The ultimate appearance of evolutionally determined characteristics is based on multiple levels of modulation and its complexity varies among organisms. The constitutional and organizational simplicity of unicellular organisms (such as prokaryotes and certain single-cell eukaryotes) allows regulatory mechanisms to be less complex. However, continuous adaptation and response to signals from the extracellular milieu is a considerably more complicated challenge for multicellular organisms in many ways. This complexity is reflected at various steps apparent during eukaryotic gene-expression.

Modulation of transcriptional activity of functional genes has been an intriguing area of research since the early 80`s, but *the disparity between the transcriptional activity of a gene and the measured level of its product has focused attention to an additional component of regulation* in many instances (1,2). This level of regulation is termed *post-transcriptional* and includes events from mRNA stability and decay to mechanisms linked to translation (3-14). The steady-state level of mRNAs depends on their rate of synthesis and cytoplasmic clearance. The latter can occur through either a translation-coupled process or regulated mRNA decay. Assuming a steady translational rate for a given mRNA, the cytoplasmic pool available for translation can substantially be altered by changes in mRNA stability. In addition to transcription, this can give rise to rapid up- or downregulation in the level of the gene product (7,8,9,15,16). It is not surprising therefore, that this mechanism gains a distinguished role in the expression of cytokines, lymphokines, certain transcription factors and proto-oncogenes, whose prompt and tightly regulated production is fundamental during normal cell growth and differentiation (17-26).

Although, it is believed that most mRNA molecules are stable, it was not quite obvious until the middle of the last decade, of how shortlived mRNAs are being destabilized in eukaryotic cells. In 1986, Kamen and Shaw documented that *many intrinsically unstable mRNAs carry specific instability determinant sequences within their highly conserved 3`-untranslated region (UTR)* (27). These authors not only provided this clue, but also established the role of such sequences in conferring instability using chimeric constructs. At that time, the functional AU-rich instability sequence elements (ARE) were characterized as specific pentamer motifs (AUUUA) embedded in tandem reiterations or clusters in a U-rich context (**Figure 1**). Large number of evidence has established the involvement of specific 3`-UTR AREs in post-transcriptional mechanisms operant in the expression of various gene products (22,23,24,28). Moreover, recently, by careful functional mapping of the proto-oncogene, *c-fos* mRNA 3`-UTR as well as by monitoring the effect of various synthetic AREs in heterologous constructs, two independent studies revealed that *the functional sequence unit within the 3`-UTR of labile mRNAs may be a nonamer, UUAUUUA(U/A)(U/A)* (29,30).

It was tempting to speculate on the potential mechanisms of action of various 3`-UTR ARE *cis* elements. It had long been suspected that a given mRNA molecule retains its intimate association with protein entities even after leaving the nuclear compartment of the cell. Indeed, the identification of many cytoplasmic messenger ribonucleoprotein particles (mRNP) highlighted the possible role of protein regulators in cytoplasmic mRNA metabolism (31,32,33). Probably the best characterized mRNA binding

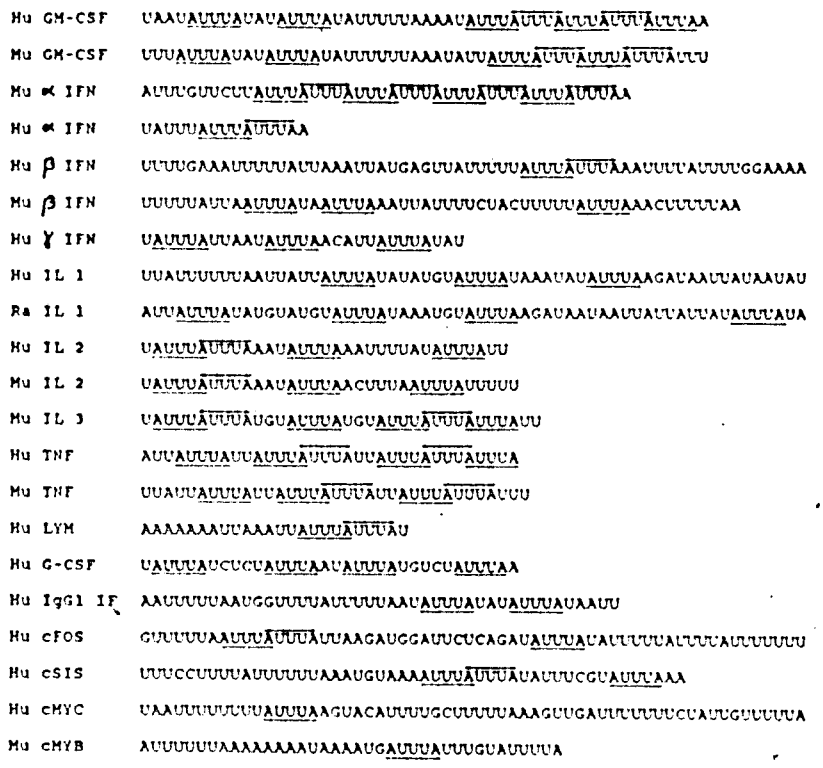


Figure 7. AU-Rich Sequences Found in the 3′ Untranslated Region of Several Lymphokine and Proto-Oncogene mRNAs

Abbreviations: Hu = human; Mu = murine; Ra = rabbit; GM-CSF = granulocyte-monocyte colony stimulating factor; αIFN = leukocyte interferon; β-IFN = fibroblast interferon; γIFN = lymphoblast interferon; IL1 = interleukin 1; IL2 = interleukin 2; IL3 = interleukin 3; TNF = tumor necrosis factor; Lym = lymphotoxin; IgG IF = IgG1 induction factor; G-CSF = granulocyte colony stimulating factor; cFOS = *fos* proto-oncogene; cSIS = *sis* proto-oncogene; c-MYC = *myc* proto-oncogene; c-MYB = *myb* proto-oncogene. The underlined/overlined sequence (AUUUA) is the largest motif common to all mRNAs shown. References: HuGM-CSF (Wong et al., 1985); MuGM-CSF (Gough et al., 1984); Mu α-Ifn (Shaw et al., 1983); Hu α-Ifn (Goeddel et al., 1981); Hu β-Ifn (Ohno et al., 1981); Mu β-Ifn (Higashi et al., 1983); Hu γ-Ifm (Gray et al., 1982); Hu IL-1, RaIL-2 (Furutani et al., 1985); Hu IL-2 (Kashima et al., 1985); MuIL-2 (Fuse et al., 1984); MuIL3 (Miyatake et al., 1985); Hu TNF, Hu LYM (Nedwin et al., 1985); MuTNF (Fransen et al., 1985); Hu G-CSF (Nagata et al., 1986); Hu IgGIF (Noma et al., 1986); Hu *c-fos* (van Straaten et al., 1983); Hu *c-sis* (Ratner et al., 1985); Hu *c-myc* (Battey et al., 1983); Hu *c-myb* (Gonda et al., 1985).

Figure 1.

proteins are members of the 5` cap-binding complex (eIF4A, eIF4E and p220) and the poly(A)-tail binding protein (PABP). The involvement of these factors in various phases of translation and possibly in mRNA decay has been well established (Reviewed in 34). It was in 1989 when Malter identified the first protein complex with specific affinity to 3`-UTR ARE sequences of various cytokine and proto-oncogene mRNAs in a human lymphocyte cell line (35). These *AU-rich sequence element binding proteins (AUBPs) specifically recognize and bind ARE in vitro.* Moreover, complex processes have been implicated in the regulation of AUBP binding to various sequences (36,37,38). These studies focused attention to the involvement of ARE-AUBP interactions in other models. Additionally, they opened several new routes to investigate critical elements in ARE/AUBP mediated mRNA turnover and/or translation, similarly to the well characterized iron-response element (IRE) - IRE-binding protein (IRE-BP) system in the metabolism of transferrin-receptor and ferritin mRNAs (Reviewed in 39). In addition to its importance in normal cell growth and differentiation, disturbance of post-transcriptional mechanisms have been proposed in a number of transformed cell types (40,41,42). In some instances, genetic alterations within critical regulatory sequences were apparent (40,42), while in other cases, the lack of such abnormalities suggested additional mechanisms, perhaps at the level of regulatory proteins (40,41).

It is well established that certain phases of cytoplasmic mRNA metabolism are coupled to cytoarchitecture. The best studied process in this respect is translation. Components of the translational apparatus have been demonstrated to associate with cytoskeletal filaments (34,43). Moreover, many mRNA subsets are translated only when anchored to the cytoskeleton (43). Disruption of the microfilament network, for instance, results in

dissociation of polysomes from the actin-based structures and yields mRNAs with impaired translatability (43). On the other hand, a large number of evidence exists, demonstrating clearly that various mRNA species are localized to and presumably translated in certain polysomal subfractions within the cell (44). Specific 3`-UTR sequences have been implicated in both mRNA localization and translation (12,13,45). *c-myc* and β-globin mRNAs are well characterized examples (Reviewed in 45). Little is known, however, whether regulation of mRNA turnover and decay involved any component linked to cytoplasmic filament networks. This question may especially be interesting in the case of lymphocytes where transient rearrangement of various cytoskeletal components is apparent during activation. The close coupling of ARE-AUBP mediated mRNA stability with translation in certain systems (11,12,13,19), as well as the above mentioned observations support the possibility of cytoskeletal involvement in specific mRNA stability.

In conclusion, during the past decade, large number of evidence has been obtained, indicating clearly the critical importance of post-transcriptional mechanisms in the regulation of eukaryotic gene-expression.

1.) Many cytokine, lymphokine, proto-oncogene and certain transcription factor genes are activated transiently at specific points of cell growth and differentiation, and their expression involves key post-transcriptional mechanisms.

2.) Transient stabilization of normally short-lived mRNAs provides a sensitive, quick and potent mechanism upon which considerable enhancement in the production of the gene product can be reached.

3.) Critical 3`-UTR sequence elements and proteins capable of specifically interacting with them have been identified and characterized in many systems. The role and importance of such components in mRNA stability and translatability as well as in localization are well established.

4.) Lymphokines are an important group of mediators and their precise production importantly relies on post-transcriptional mechanisms, including selective mRNA turnover regulation. Therefore, various lymphoid cell systems represent a valuable tool which can be applied to uncover key aspects of this potent level of regulation in eukaryotic gene expression.

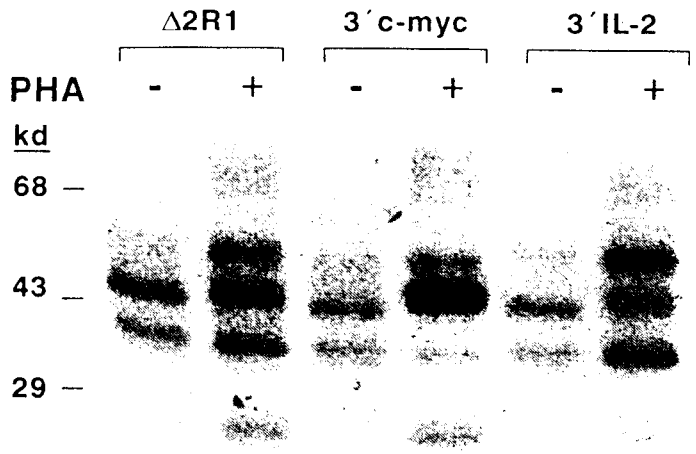
5.) The strong interaction of translational machinery with the cytoskeleton as well as the involvement of cytoarchitecture in sorting and localization of various polysomal assemblies suggest that elements of cytoplasmic mRNA metabolism other than translation may also be linked to cytoskeletal organization and function.

Chapter 2: PROBLEM AND PRELIMINARY RESULTS

Lymphokines are multifunctional regulators of lymphoid cell growth and differentiation as well as many immunobiological functions. These transiently expressed proteins are encoded by mRNAs with short half lives ($T_{1/2}$<15-30 min). During early phases of lymphocyte activation, a several-fold increase in the transcriptional rate of many lymphokine genes can be detected (6), leading to the production of a specific protein profile. From our own studies as well as from work of others, it has been calculated that the transcriptional activity of lymphokine genes, such as IL-2, γ-IFN or TNF-α cannot account solely for the amount of lymphokines produced (6,17). This prompted us to search for potential mechanisms which could provide explanation for this disparity. We analyzed specific lymphokine mRNA turnover in activated PBMCs and T lymphocytes. Indeed, we have shown that following transcriptional upregulation, lymphokine mRNAs are selectively stabilized in a specific sequence upon activation (17,46). This was in good agreement with observations of others (6). It also explained of how activated lymphocytes encounter the remarkable challenge of upregulating their lymphokine expression within a relatively short time.

Based on the results of the search for key elements responsible for regulated mRNA stability outlined above, our aim was to see if activated lymphocytes contained cytoplasmic proteins with AUBP activity and whether this could be correlated with lymphokine mRNA stability. We detected many cytoplasmic proteins with AUBP activity in resting and activated lymphocytes (**Figure 2**). Moreover, we showed that the level of these proteins was increased by activation, suggesting their involvement in activation-induced lymphokine mRNA stabilization (47).

Additional important observations came from our studies conducted within a somewhat different context. Following transcription, pre-mRNA



Δ2R1 5' T7- GGAUCCAUUUAUUUAUUUAUUUAAGCUUGG-3'

Δ2H3 5' T3- CCAAGCUUAAAUAAAUAAAUAAAUGGAUCC-3'

3'IL-2 5' T7- AGGCCUUCUAUUUAUUUAAUAUUUAAAUUUUA
UAUUUAUU-$(N)_{120}$-CACUUAUUUAUCCCAAAUAU
UUAUUA-$(N)_{40}$-AAAACUAUUUAAUAAAUUUGAUA
AAUAU- $(A)_{12}$-C-$(A)_{11}$-3'

3'IL-2AS 5' T3- $(U)_{11}$-G-$(U)_{12}$-AUAUUUATCAAAUUUAUUAAAUAGUUU
UACUAACCAAUCUACAUAGAUACUAUAUUUAACAUU-
CAACA-$(N)_{80}$-UAUUUAUAGUUUUUUGAUUAAGA-$(N)_{40}$
AAAUAUAAAAUUUUAAAUAUUUAAAUAAAUAGAAGGCCU

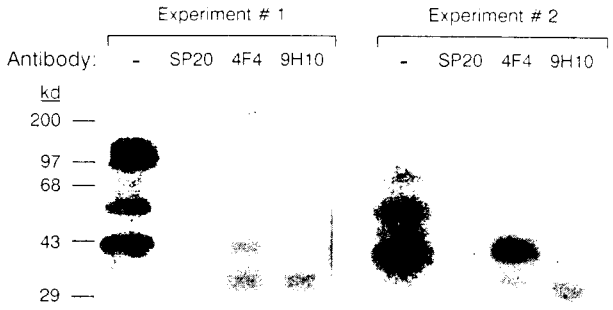
3' c-myc 5' SP6- UGAUCAAAUGCUUCCUCACAACCUUGGCUGAAGUC
UUGAGACUGAAAGAAUUUAAGCCAUAAUGUAAAACU
GCCUCAAAUUGGAACUUUGGGCAUAAAAGAACUUU
UUUAUGCUUACCAUCUUUUUUUUUCUUUAACAGAUU
UGUAUUUAAGAAUUGUUUUUAAAAAAUUUUAAGAU
UUACACAAUGUUUCUCUGUAAAU-3'

TGF-β2 5' SP6-$(N)_{574}$-GAACCUGGGAUUUAAAAUA

AUUUA-binding proteins detected in human resting and PHA-activated PBMC. *Upper panel*, cytoplasmic lysates (20 μg of protein) from human lymphocytes cultured 24 h ± PHA were incubated with 8×10^4 cpm of indicated ^{32}P-RNA probes, UV cross-linked, and RNase digested before analyzing under reducing conditions by SDS-PAGE. *Lower panel*, sequence of RNA probes with AUUUA sequences *underlined* are shown.

Figure 2.

molecules promptly assemble with heterogeneous nuclear ribonucleoproteins (hnRNP) and form dynamic complexes during nuclear pre-mRNA processing and splicing (48). The intriguing work by Dreyfuss et al. demonstrated that one class of hnRNP proteins, the 36-kDa hnRNP A1 molecule shuttles between the nucleus and cytoplasm in response to Act D treatment (49). This, together with our previous observation that, among others, a 36-kDa AUBP enriches in cytoplasmic fractions of activated and Act D treated cells, prompted us to examine the possible identity of these two proteins. We could clearly identify the 36-kDa protein as being hnRNP A1 as well as a 43-kDa AUBP as hnRNP C in lymphocytes and implicated their role in selective lymphokine mRNA metabolism (47). These data, for the first time, demonstrated members of the most abundant nuclear protein family in connection with selective mRNA metabolism in the cytoplasm, and suggested a role clearly distinct from their previously known nuclear functions (**Figure 3**).



Immunoprecipitation of AUUUA-binding proteins with monoclonal antibodies for hnRNP C and A1 proteins. Human lymphocyte cytoplasmic lysates (PHA-activated for 8 h) were incubated with ^{32}P-Δ2R1, UV cross-linked, and digested with RNase. The RNA-protein complexes were immunoprecipitated with protein A-Sepharose beads following incubation with SP20 (negative control antibody), 4F4 (anti-hnRNP C), or 9H10 (anti-hnRNP A1) ascites, boiled in SDS sample buffer, and analyzed by SDS-PAGE and autoradiography for 7 days. Untreated lysates are shown as shorter exposures, with the 36-kDa AUBP poorly visualized.

Figure 3.

These findings raised a number of pertinent questions as well as provided a fertile soil for further studies to uncover additional mechanisms which may operate during post-transcriptional regulation of lymphokine production. The raised questions are outlined below, and the results, obtained from these studies are presented in the Thesis in the form of two published papers and a submitted manuscript.

1. The mechanisms of how ARE-AUBP interactions contribute to selective mRNA turnover and hence, to proper post-transcriptional regulation of gene-expression is not completely understood.

1.1. How are ARE and AUBPs affected in a system where a known abnormality of lymphokine production is observed?

1.2. How do lymphokine ARE and AUBPs interact during changes in mRNA stability? What is the nature of such interactions?

1.3. Can specific changes in *in vitro* ARE-AUBP interactions be correlated with mRNA stability *in vivo*?

Enhanced stability of interleukin-2 mRNA in MLA 144 cells: Possible role of cytoplasmic AU-rich sequence-binding proteins (1994) ***J. Biol. Chem., 269:*** *5377-5383*

2. Although, many valuable information have been derived from *in vitro* experimental models, no attempts were made to investigate the nature of ARE-AUBP interactions *in vivo*.

2.1. Is there a suitable method which could be applied to monitor *in vivo* interactions between various ARE and AUBPs relevant to physiological regulation?

2.2. Are there detectable changes in the association of various AUBP with ARE *in vivo* in response to various stimuli, such as activation?

2.3. Do cytoplasmic AUBPs show specific distribution between functional compartments, such as various polysomal subfractions?

Combined application of in vivo UV-crosslinking and in vitro label transfer in the examination of AU-rich sequence binding protein - RNA interactions (1995) ***Cell Biol. Internatl., 19:*** *791-801*

3. A number of evidence suggest that cytoskeleton-based subcellular targetting and localization are apparent elements in cytoplasmic mRNA metabolism, including translation.

3.1. Does cytoarchitecture influence mRNA stability in lymphocytes, where prominent rearrangement of cytoskeletal elements is observed during activation?

3.2. Can specific association of lymphokine mRNAs and/or AUBPs be detected with any cytoskeletal elements?

3.3. Do the association of ARE-AUBP complexes and their normal function require intact cytoskeletal components?

Interaction of AU-rich sequence binding proteins with actin: Possible involvement in lymphokine mRNA turnover (1996) submitted to ***J. Biol. Chem.***

Chapter 3: EXPERIMENTAL APPROACHES

In this Chapter, I highlight the most important experimental techniques used in these studies, although, for additional details, I refer to the Materials and Methods sections of the presented publications.

Cell cultures. Peripheral blood mononuclear cells (PBMCs) were derived from healthy individuals using the *Ficoll Paque gradient separation* method. IL-2 lymphoblasts were obtained by culturing PBMCs in the presence of recombinant IL-2. MLA-144 cells were from ATCC.

Determination of lymphokine gene transcription and lymphokine mRNA stability. Lymphokine gene-transcription was monitored by the *nuclear "run-on" technique.* A transcriptional "mix" was generated by adding labeled precursors to isolated nuclei. Following DNase and Proteinase K treatment, RNA was extracted and hybridized on a *slot blot* with immobilized and linearized dsDNA plasmids containing various lymphokine sequences. Hybrids were visualized by autoradiography. Schematic illustration of the technique is presented in **Figure 4**. For determination of RNA stability, cells were exposed to transcriptional inhibitors, such as Act D or DRB, which is more specific for RNA Polymerase II. At various time points, total cellular RNA was extracted by the single-step guanydium-thyocianate phenol-chloroform method (50). Poly (A)+ RNA was prepared using *oligo-d(T) column chromatography.* Steady-state mRNA levels as well as mRNA turnover was analyzed by *Northern blotting*, using formaldehyde-agarose gels. Probes were obtained by generating labelled ssDNA from templates encoding for various lymphokine ORF sequences using the *random priming* method (51). Probed blots were exposed to X-ray film and visualized by autoradiography.

Label transfer experiments. Cytoplasmic lysates were generated by *sequential detergent fractionation* (43). Triton X-100-soluble and Triton X-

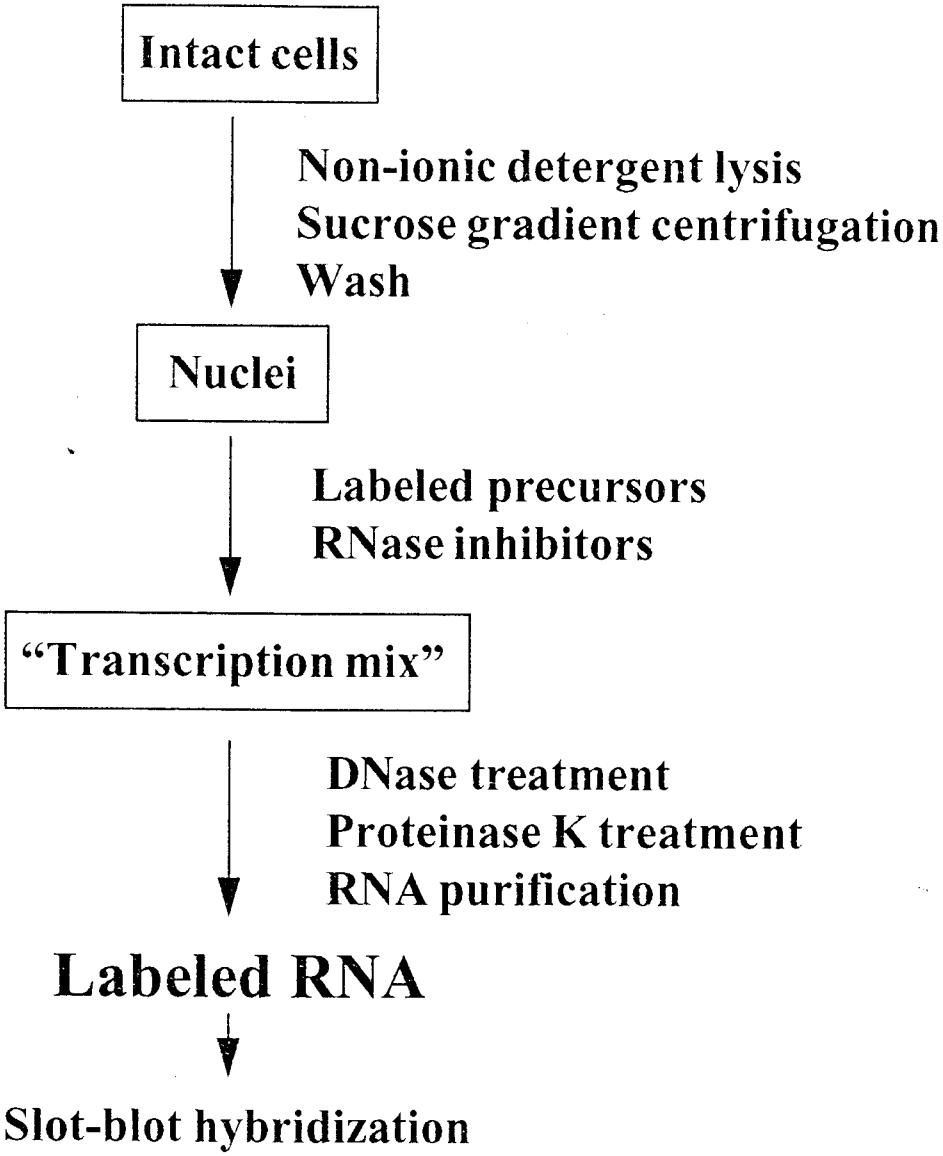


Figure 4. *Nuclear "run-on" assay*

100-insoluble, but Tween-20- and Desoxycholate-soluble subfractions were obtained and incubated with RNA probes containing 3'-UTR ARE sequences of various lymphokine mRNAs. RNA probes were synthesized in *in vitro transcription* reactions in the presence of labeled precursors. Formed complexes were fixed by UV light, exposed to RNase treatment and separated on SDS-PAGE. Gels were visualized by autoradiography. **Figure 5** schematically outlines the main steps of this method.

Western and Northwestern analyses. Subcellular extracts were separated on SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were probed by monoclonal antibodies for Western blotting and visualized by the ECL chemiluminescence method, whereas for Northwestern analysis, membranes were exposed to labeled RNA probes and analyzed by autoradiography.

In vivo UV-crosslinking experiments. This technique was developed in our laboratory and has proven to be capable of monitoring ARE-AUBP associations *in vivo* (52). Intact cells were exposed to monochromatic (λ=254 nm) UV light. UV irradiation covalently fixes protein-nucleic acid links which are in close enough proximity at the time of UV-exposure (53,54,55). Subcellular fractions were then obtained and analyzed in a label transfer assay. The identity of *in vivo* formed complexes as well as the intensity of their RNA-protein associations are judged in the label transfer assay by the binding profile and the degree of decrease in the signal given by the selected RNA probe, respectively. Complexes were separated on SDS-PAGE and visualized by autoradiography. **Figure 6** illustrates the principle of this method.

Preparation of polysomal subfractions. This procedure was based on the method, described by Hesketh et al. (44). Briefly, cells were lysed to obtain

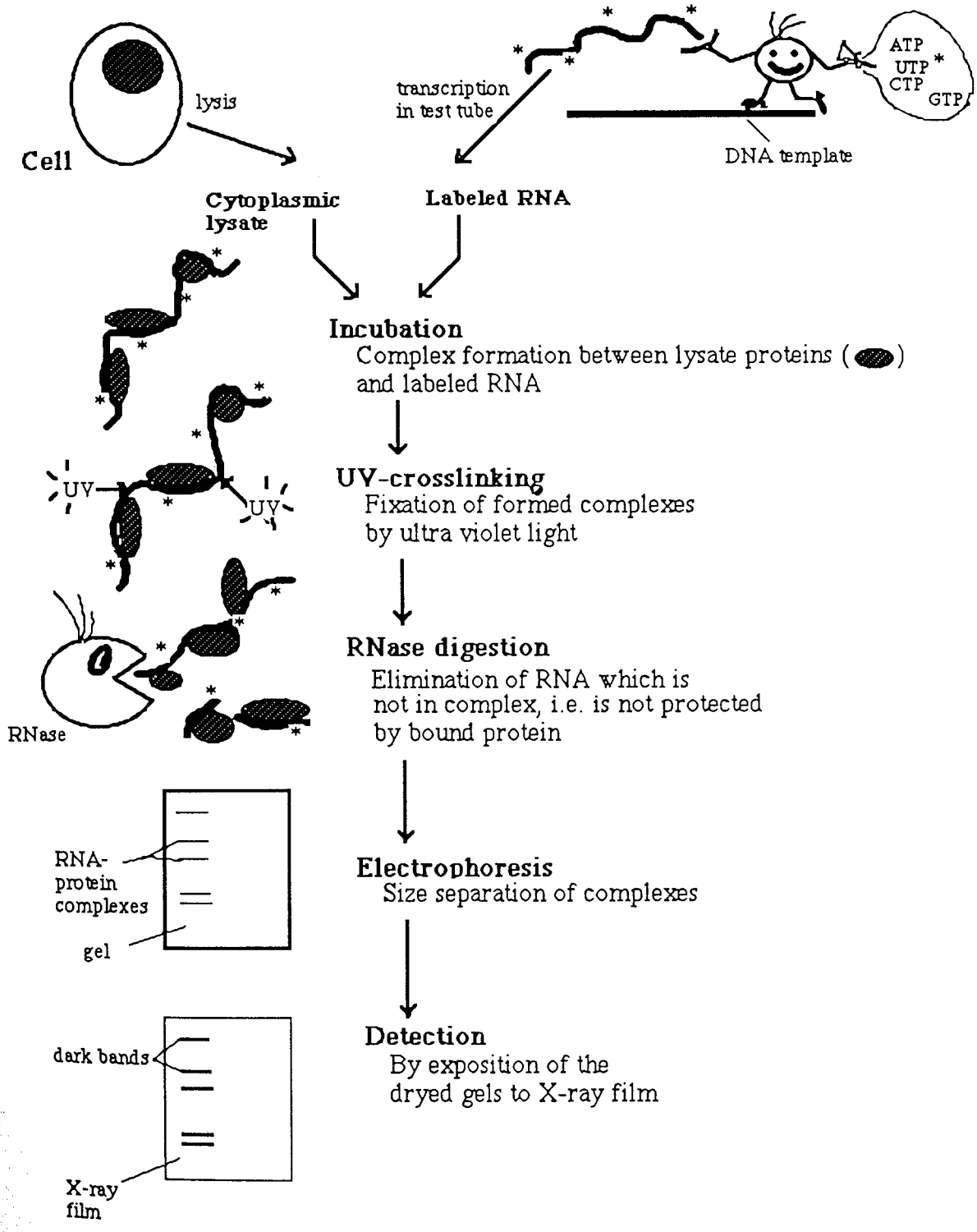


Figure 5. ***Label transfer assay***

cytoplasmic subfractions using *sequential salt-detergent extraction* steps. Fractions were overlayered and separated on discontinuous sucrose gradients by ultracentrifugation. Polysomal pellets were collected, washed and analyzed for AUBP activity in a label transfer assay.

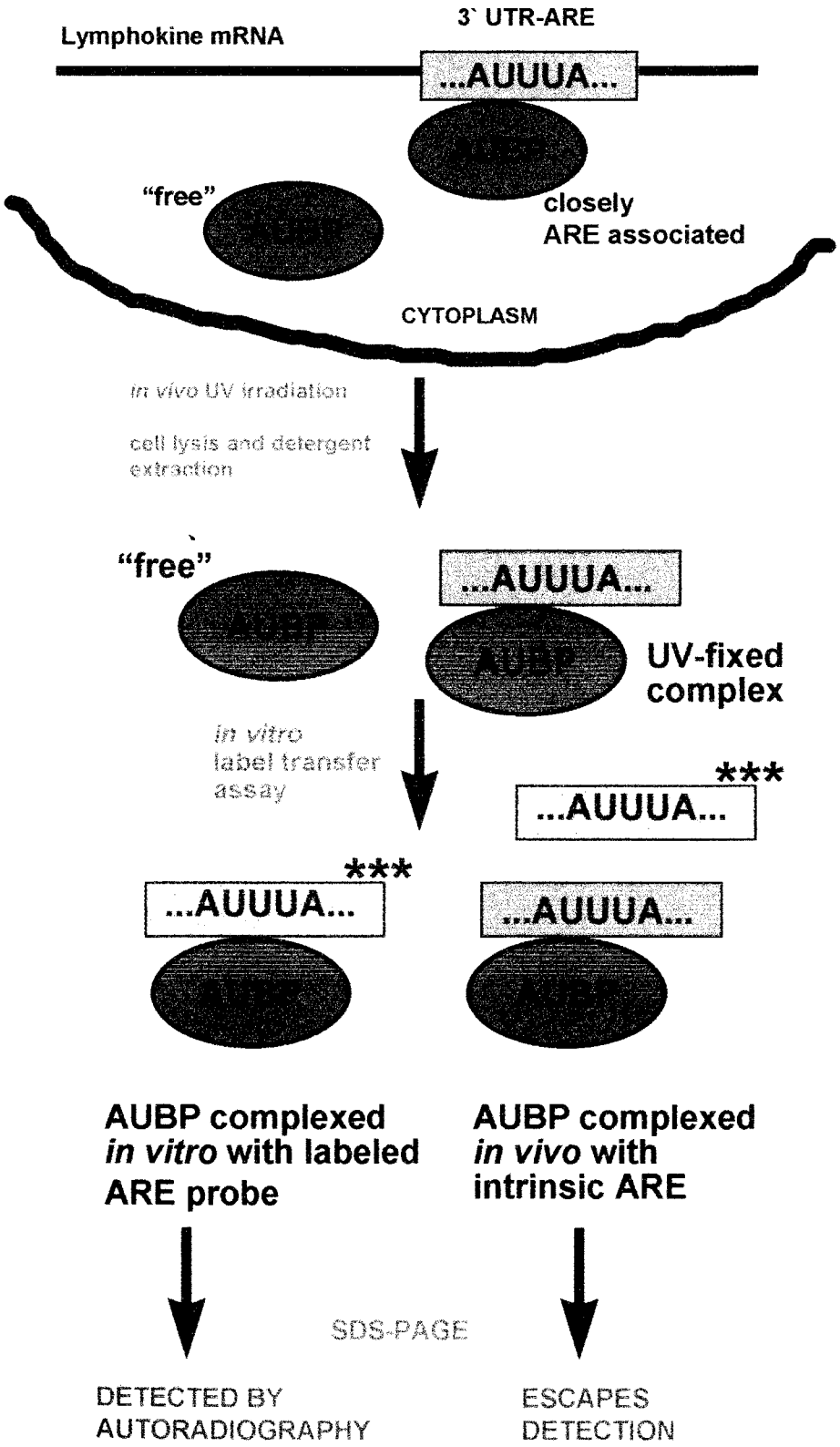


Figure 6. In vivo UV-crosslinking technique

Chapter 4: RESULTS AND DISCUSSION

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Enhanced Stability of Interleukin-2 mRNA in MLA 144 Cells

POSSIBLE ROLE OF CYTOPLASMIC AU-RICH SEQUENCE-BINDING PROTEINS*

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The MLA 144 gibbon T cell line is infected with a type C retrovirus and constitutively expresses interleukin-2 (IL-2) and granulocyte macrophage colony-stimulating factor (GM-CSF). IL-2 mRNA levels are 10-fold more abundant than GM-CSF in these cells. Comparable transcriptional rates for these lymphokines suggested the involvement of post-transcriptional mechanisms in selective IL-2 mRNA accumulation. IL-2 mRNA is exceptionally stable in MLA cells with a $t_{1/2}$ of more than 8 h. The presence of reiterated AUUUA sequences in the 3′-untranslated region (UTR) has been shown to confer mRNA lability. The provirally altered MLA IL-2 allele encodes an mRNA in which three AUUUA motifs have been deleted. Six major cytoplasmic proteins bound *in vitro* transcribed RNA probes containing sequences from the 3′-UTR of normal human IL-2 (3′-IL-2), GM-CSF (Δ2R1), and the virally altered MLA IL-2 (3′-IL-2 PV) mRNA. Increased binding of these proteins to 3′-IL-2 PV was observed relative to 3′-IL-2 or Δ2R1. Northwestern blotting demonstrated similar differential ability of a 36- and 43-kDa protein to bind, as well as showed that these proteins colocalized by immunoblotting as hnRNP A1 and C, respectively. These findings suggest a direct correlation between differential binding of cytoplasmic proteins to AU-rich 3′-UTRs *in vitro* and lymphokine mRNA stability *in vivo*.

Post-transcriptional processes provide an important level of regulation in eukaryotic gene expression in response to various external stimuli (1, 2). Biologically active molecules (cytokines, proto-oncogenes, lymphokines) important in cell growth and differentiation are frequently encoded by mRNA that exhibit rapid cytoplasmic turnover (half-life = $t_{1/2}$ < 30 min) (3–5). Reiterated AUUUA pentamers (ARE) found within the 3′-untranslated region (UTR)[1] of these intrinsically labile mRNAs have been shown to confer mRNA lability using heterologous gene constructs (6, 7). These ARE appear to function as *cis*-regulatory elements capable of modulating both the turnover and translation of specific mRNAs (6–8). Cytoplasmic proteins that specifically bind to ARE of the 3′-UTR of lymphokines (9), cytokines (10), and proto-oncogenes (11, 12) have been identified as potential *trans*-acting factors which transduce this activity. Nevertheless, direct functional correlations between specific cytoplasmic protein binding to an ARE and mRNA turnover are lacking. This correlation may be of importance in understanding the disordered cell growth and differentiation that characterize neoplastic transformation. Considerable evidence exists to suggest that ARE-dependent mRNA turnover is important in tumorigenesis (13–15). Additionally, enhanced proto-oncogene or growth factor mRNA stability is observed frequently in neoplastic cells (16, 17). Thus, evolution and/or maintenance of the transformed state might possibly be mediated through enhanced expression of active growth factors or proto-oncogenes. In this paper, we describe a cell line in which abnormal growth factor mRNA stability is observed and provide a direct correlation between AUBP-ARE interactions *in vitro* with cytokine mRNA stability *in vivo*.

Constitutive production of biologically active lymphokines, such as interleukin 2 (IL-2) and granulocyte macrophage colony-stimulating factor (GM-CSF), is a characteristic feature of the cell line MLA 144 (18). These cells were derived from lymphoid tumor tissue and are infected with a type C retrovirus, gibbon ape leukemia virus, which has been associated with several hematopoietic neoplasms (19). Constitutive IL-2 gene transcription occurs in the presence of two retroviral insertions within one allele of the MLA IL-2 gene (20). A viral 5′ long terminal repeat (LTR) is found in the 5′-flanking region in the antisense orientation, about 1200 bases upstream from the coding region. Transcriptional analysis demonstrated that this gibbon ape leukemia virus sequence has weak enhancer activity (21). Additionally, a proviral insertion with an internal 3.25-kb deletion is present within the 4th exon of the IL-2 gene. This insertion results in a 1.1-kb mRNA (≈200 bases longer than the native IL-2 message) in which the coding region and the first 100 bases of the 3′-UTR are that of IL-2, and the remaining 495 bases are that of the LTR of the provirus. This 3′ proviral integration event deletes three of the seven AUUUA pentamers of the IL-2 mRNA, but preserves the AU-rich region containing the remaining four ARE, 43 bases 3′ to the coding region (20). At the site of proviral insertion, there is also a deletion of four bases from the native IL-2 gene.

We observed that IL-2 mRNA is more abundant than GM-CSF despite comparable rates of transcription in MLA 144 cells. Message stability studies revealed IL-2 mRNA to be exceptionally stable with a half-life ($t_{1/2}$) of more than 8 h. In contrast, GM-CSF mRNA turnover was rapid ($t_{1/2}$ < 30 min), indicating that a generalized derangement of ARE-dependent mRNA turnover was not operant. Six major cytoplasmic proteins (36, 41, 50, 70, 85, and 90 kDa) from MLA 144 cells were

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[1] The abbreviations used are: UTR, untranslated region; IL-2, interleukin-2; GM-CSF, granulocyte macrophage colony-stimulating factor; LTR, long terminal repeat; ActD, actinomycin D; DRB, 5,6-dichloro-1-β-ribouranosylbenzimidazole; PHA, phytohemagglutinin; PIPES, 1,4-piperazinediethanesulfonic acid; cpm, counts/minute; PAGE, polyacrylamide gel electrophoresis; hnRNP, heteronuclear ribonucleoprotein.

observed to bind to the ARE of provirally modified IL-2 mRNA to a greater degree than to those found in native IL-2. Similar data were observed with cytoplasmic lysates of activated human T lymphocytes. Characterization of the proviral sequences necessary for altered AUBP binding were performed. Enhanced binding of the provirally modified IL-2 3′ UTR was apparent by Northwestern blotting, which permitted colocalization of the 36- and 43-kDa AUBPs with hnRNP A1 and C, respectively. Recombinant human hnRNP A1 and C1 proteins demonstrated comparable patterns of RNA probe binding. These data suggest a relationship between AUBP and hnRNP binding *in vitro* and ARE-dependent mRNA turnover *in vivo*. Moreover, these findings indicate that the context in which ARE exist appears to be of importance in determining AUBP binding *in vitro*.

MATERIALS AND METHODS

Reagents—Actinomycin D (ActD) and 5,6-dichloro-1-β-ribofuranosylbenzimidazole (DRB) were purchased from Sigma. ActD was kept at 4 °C as a 5 mg/ml stock solution whereas DRB was made up freshly before use. Phytohemagglutinin (PHA) was obtained from Wellcome Reagent Ltd., Beckenham, United Kingdom. [^{32}P]UTP (3000 Ci/mmol) was purchased from Amersham Corp., and unlabeled nucleotides were obtained from Boehringer Mannheim. Recombinant human hnRNP A1 and C1 as well as antibodies against these proteins were generously provided by Dr. Gideon Dreyfuss, whereas recombinant human La protein was a generous gift of Dr. William St. Clair.

Cell Culture—MLA 144 cells (American Type Culture Collection, Rockville, MD) were maintained in RPMI-1640 medium (JHR Biosciences, Lenexa, KS) supplemented with 8% heat-inactivated (56 °C, 30 min) fetal bovine serum (Flow Laboratories, McLean, VA) at a density of 0.5–1 × 10^6 cells/ml and subcultured every second day. Human lymphocytes were separated on discontinuous Ficoll-Hypaque gradient from leukapheresed blood obtained from healthy volunteers and cultured in 8% fetal bovine serum-RPMI-1640 medium in the presence of 1 μg/ml PHA for different periods of time.

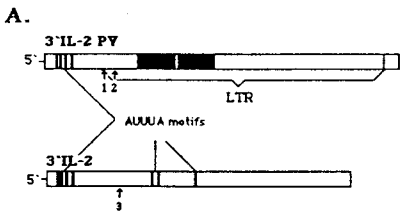
Transcriptional Inhibition Experiments, RNA Isolation, and Detection of IL-2 and GM-CSF Transcripts—0.5–1 × 10^8 cells were seeded in 250-ml tissue culture flasks (Falcon) at 10^6/ml density and treated either with ActD (5 μg/ml) or DRB (100 μM) for different periods of time. This concentration of DRB was shown to inhibit [^{3}H]uridine incorporation by MLA 144 cells over 95% within 5 min while having no effect on cell viability for up to 6–8 h. Cells were harvested, and total cellular RNA was isolated by the single step guanidium thiocyanate-phenol-chloroform extraction method (22) with a modification of increasing the amount of β-mercaptoethanol (Sigma) from 0.1 to 0.7 M in the 5 M guanidium thiocyanate (Fluka Biochemica) denaturing solution. Poly(A)$^+$ RNA was prepared by oligo(dT) column (Collaborative Biomedical, Bedford, MA) chromatography. For Northern blot analyses, equal amounts (5–10 μg) of RNA samples were size fractionated by 0.8% agarose-formaldehyde gel electrophoresis, blotted on Hybond nylon membrane (Amersham Corp.) overnight in 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). Membranes were air dried and baked at 80 °C, 2 h *in vacuo*, then prehybridized for at least 12 h in 50% formamide, 0.8 M NaCl, 0.1 M PIPES, 0.1% Sarkosyl, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, and 200 μg/ml sheared salmon sperm DNA at 42 °C. RNA was detected by hybridization of the membranes at 42 °C overnight in prehybridization mix containing 10% dextran sulfate and 1 × 10^6 cpm/ml of [^{32}P]dCTP-labeled cDNA probes generated by the random priming method (23). Blots were then washed three times at 56 °C in 0.1 × SSC containing 0.02% sodium pyrophosphate and 0.5% Sarkosyl. Blots were air dried and exposed to Kodak XOmat film at −80 °C.

Nuclear Run-on Analysis—Nuclei were prepared by lysing 2–3 × 10^8 cells in a buffer (10 mM HEPES, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$) containing 0.05 % Nonidet P-40 detergent and centrifuged at 1000 × *g* through a 30% sucrose cushion at 4 °C. Nuclei were then resuspended in 50 mM HEPES, pH 7.9, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA at a density of 10^8/ml, snap frozen in liquid nitrogen in 100-μl aliquots, and stored at −80 °C. *In vitro* transcription reactions were carried out at room temperature by adding equal volume (100 μl) of two times transcription buffer (50 mM HEPES, pH 7.9, 120 mM KCl, 20 mM dithiothreitol, 30 mM β-mercaptoethanol, 50 mM magnesium acetate, 2 mM $MnCl_2$, 1 mM EDTA, 8 mM phosphoenolpyruvate, 6 μg/ml pyruvate kinase, 2 mM fructose-1,6-diphosphate, 1% Tween-20, 2 mM thymidine diphosphate, 1 mM ATP, CTP, and GTP each, 10 μM UTP, 1000 units/ml RNasin, and 0.4 mg/ml heparin) to 100 μl of nuclear suspension and incubated for 15 min at room temperature in the presence of 125 μCi of [^{32}P]UTP. Following DNase and proteinase K treatment, the mixture was phenol-chloroform extracted twice and precipitated in ethanol. Labeled RNA was purified by size-exclusion column chromatography (Bio-Spin 30, Bio-Rad). Following prehybridization in a mix containing 50 mM sodium phosphate, 50% formamide, 500 μg/ml yeast tRNA, 10% SDS, 0.8 M NaCl, 0.1 M PIPES, 0.1% Sarkosyl, 0.1% Ficoll, 0.1% polyvinylpirrolidone, and 0.1% bovine serum albumin, results were detected by linearized plasmids containing the cDNA blotted onto Hybond nylon membranes (5 μg/slot) using a slot-blot apparatus (Schleicher & Schuell). The purified run-on transcripts were added directly to the prehybridization mix (10^7 cpm/ml final) and incubated at 42 °C for 48–72 h. After washing three times in 2 × SSC containing 0.02% sodium pyrophosphate and 0.5% Sarkosyl at 56 °C, membranes were dried and exposed to Kodak X-Omat film at −80 °C.

Cytoplasmic Lysate Preparation—Cytoplasmic lysates were prepared as described (24) with minor modifications. Briefly, 20 × 10^6 cells were washed in ice-cold serum-free medium, pellets were resuspended in buffer A containing 10 mM PIPES, pH 6.8, 100 mM KCl, 2.5 mM $MgCl_2$, 300 mM sucrose, 1 mM phenylmethylsulfonyl fluoride and lysed on ice for 3 min by addition of 1% Triton X-100. Following centrifugation for 3 min at 900 × *g*, supernatants were collected, aliquoted, and frozen immediately. Exclusion of 1 mM $CaCl_2$ from the lysis buffer did not influence the detected levels of cytoplasmic AUBPs in this system. The Triton-insoluble pellets (cytoskeleton and associated components) were extracted in buffer B containing 10 mM HEPES, pH 7.4, 15 mM NaCl, 1.5 mM $MgCl_2$, 1% Tween-20, 0.5% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride for 3 min on ice. Supernatants (referred to as INSOL) were collected after centrifugation with 1200 × *g* for 3 min at 4 °C.

RNA Probes and AUBP Assay—All the RNA probes in our experiments were transcribed with the T7 or T3 RNA polymerases (as indicated in Fig. 1*B*) from cDNA templates that had been subcloned into the pT7/T3-α19 vector and prepared for *in vitro* transcription as follows. A 338-base 3′-IL-2 probe was generated from an *Eco*RI linearized plasmid which contained the 3′-UTR (270 base pairs) of the human IL-2 cDNA downstream from the *Stu*I restriction site and therefore contained. The 126-base 3′-IL-2/B probe was transcribed from the same plasmid which was linearized with *Bgl*I. 3′-IL-2 PV, 3′-IL-2 PV/P, and 3′-IL-2 PV/E were transcribed from a plasmid that contained a 565-base pair fragment of the original retrovirally modified MLA gibbon IL-2 cDNA (CM-9) downstream from the *Stu*I site (20) that had been digested with *Bgl*I and *Bam*HI, *Pst*I, and *Eco*RV prior to transcription, yielding RNA probes with 597, 96, and 171 bases in length, respectively. The 90-base Δ2R1 probe was generated by the transcription of an *Eco*RI linearized plasmid that contained a 30-base pair portion of the GM-CSF 3′-UTR containing four consecutive AUUUA pentamers (10). The AUUUA motifs in each of these RNA probes are indicated in Fig. 1*B*. Comparable AUBP binding intensities were observed with 3′-IL-2 PV and its truncated forms that had been transcribed with T7 RNA polymerase from appropriate constructs, thereby excluding any potential contribution of the MCS segment encoded in the transcript in altering binding. ^{32}P-Labeled probe mRNAs (specific activity > 10^8 cpm/μg RNA) were generated by *in vitro* transcription where 50 μCi of [^{32}P]UTP (3000 Ci/mmol), 0.0125 mM UTP, and 2.5 mM each ATP, CTP, GTP were present. 8 × 10^4 cpm probe RNAs (≈1–15 fmol) were incubated with 2.5–10 μg of cytoplasmic lysate protein or 0.05 or 0.25 μg of recombinant C1 and A1 or La proteins, respectively, in 12 mM HEPES, pH 7.9, 15 mM KCl, 0.2 μM dithiothreitol, 0.2 μg/ml yeast tRNA, and 10% glycerol for 10 min at 30 °C. Cold competitors where needed were added simultaneously to the binding reaction tubes. Protein-nucleic acid complexes were UV cross-linked on ice using Stratagene UV Stratalinker model 1800 or 2400 (5 min, 3000 microwatts/cm^2 or 3.5 min, 4000 microwatts/cm^2), then exposed to RNase digestion (15 units of RNase T1 and 30 μg of RNase A/sample) for 15 min at 37 °C. Samples were then separated by 15% SDS-PAGE under denaturing conditions, and gels were dried and analyzed by autoradiography.

Northwestern Analysis—15 μg of MLA 144 lysate protein was loaded and separated by 15% SDS-PAGE under reducing conditions. Gels were then equilibrated in transfer buffer (TB, 48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2) for 15 min at room temperature and electrotransferred onto Hybond C nitrocellulose membrane (Amersham Corp.) for 25 min at room temperature at constant 15 V using a semidry transfer system (Bio-Rad). Membranes were soaked in PBS and hybridized in solution A (10% glycerol, 15 mM KCl, 12 mM HEPES, 0.2 μg/ml yeast tRNA, 0.2 μM dithiothreitol, pH 7.9) in the presence of 8 × 10^5 cpm/ml each of the *in vitro* transcribed RNA probes indicated in Fig. 5, for 30 min at room temperature. Membranes were then washed gently three



B.

3'IL-2	5' -CCCU-n30-CUAUUUAUUUAAAUAUUUAAAUUUUAUAUUUA-UUG-n110-ACUUAUUUAUCCCAAAAUAUUUAUUA-n45-AACUAUUUAAUA-n77 (T7)
3'IL-2/B	5' -CCCU-n30-CUAUUUAUUUAAAUAUUUAAAUUUUAUAUUUA-UUG-n75-CUUUUUGUAAG (T7)
3'IL-2 PV	5' - GGGA-n10-CUAUUUAUUUAAAUAUUUAAAUUUUAUAUUUA-UUG-n42-AGAGCUGCAGUAAGGCC-n486 (T3)
3'IL-2 PV/P	5' -GGGA-n10-CUAUUUAUUUAAAUAUUUAAAUUUUAUAUUUA-UUG-n30-UAUUAUUCUUAAAGAGC (T3)
3'IL-2 PV/E	5' -GGGA-n10-CUAUUUAUUUAAAUAUUUAAAUUUUAUAUUUA-UUG-n105-CAGGGCCAAACAGGAU (T3)
Δ2R1	5' -CCCU-n40-CCAUUUAUUUAUUUAUUUAAGCUU-n22 (T7)

FIG. 1. *A*, schematic illustration of the 3′-UTRs of MLA 144-IL-2 and normal human T lymphocyte IL-2 mRNA. A 495-nucleotide long viral LTR is inserted within this portion of the MLA IL-2 mRNA with the deletion of three of the seven AUUUA pentamers (*dark bars*). There are two identical 94-nucleotide long direct repeat elements within the LTR (*dark areas*). *Arrows* indicate unique restriction sites where the cDNA was cut in order to generate templates for transcription of the corresponding RNA probes *in vitro* (*1*, *Pst*I → 3′-IL-2 PV/P; *2*, *Eco*RV → 3′-IL-2 PV/E; *3*, *Bgl*I → 3′-IL-2/B). *B*, sequences of *in vitro* transcribed RNA probes with the AUUUA motifs outlined within their AU-rich 3′ regions. The fragments of non-vector origin bordering the first four AUUUA motifs for six bases upstream and 28 bases downstream of the 3′-IL-2 or 3′-IL-2 PV and related probes are identical. A four-base deletion is then found in 3′-IL-2 PV (GUAA in 3′-IL-2) and related probes, with identity resumed for another 12 bases prior to the beginning of the LTR. RNA polymerases used to generate the RNA probes are indicated in *parentheses*.

times in solution B (15 mM KCl, 12 mM HEPES, pH 7.9) at room temperature, air dried, and analyzed by autoradiography.

Western Blotting—Cytoplasmic lysates from MLA 144 cells were size separated by denaturing SDS-PAGE and electrotransferred onto nitrocellulose membranes (Schleicher & Schuell) as described for Northwestern analysis. Membranes were incubated with either anti-hnRNP C antibody (4F4) or anti-hnRNP A1 (4B10) and visualized by using the ECL chemiluminescence detection method (Amersham Corp.).

RESULTS

Comparison of IL-2 and GM-CSF mRNA Expression in MLA 144 Cells—Comparison of IL-2 and GM-CSF mRNA levels in MLA 144 cells demonstrated markedly higher levels of IL-2 mRNA relative to GM-CSF (Fig. 2*A*, *lane 1*). Northern blot analysis indicated additionally that IL-2 mRNA was readily detectable with total cellular RNA preparations while GM-CSF mRNA required analysis of poly(A)$^+$ RNA for detection (Fig. 2, *A* and *B*). This finding suggested at least a 10–100-fold greater level of IL-2 mRNA relative to GM-CSF. The role of gene transcription was analyzed to define the potential mechanism behind the differential levels of IL-2 and GM-CSF mRNA accumulation in MLA cells. Nuclei were assayed for *in vitro* rates of transcription by nuclear run-on analysis (Fig. 2*C*). Equivalent rates of constitutive transcription of both IL-2 and GM-CSF genes were present, suggesting that transcription could not account for the increased levels of IL-2 mRNA relative to GM-CSF in MLA 144 cells.

Different Rates of IL-2 and GM-CSF mRNA Turnover—The

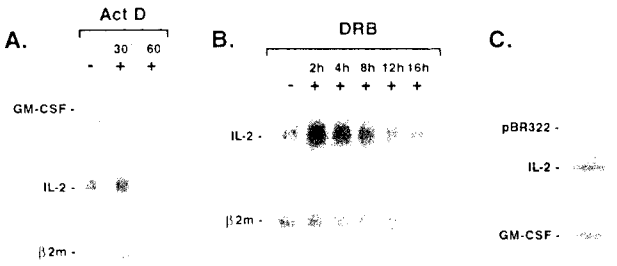


FIG. 2. **Northern blot analysis of IL-2 and GM-CSF mRNA production by MLA 144 cells.** *A*, following transcriptional inhibition by ActD and RNA extraction, 5 μg of poly(A)$^+$ RNA was analyzed in each lanes. GM-CSF mRNA is very unstable ($t_{1/2}$ < 15–30 min) while IL-2 mRNA is exceptionally stable with $t_{1/2}$ > 8 h as determined by Northern blotting of 15 μg/lane total cellular RNA extracted from cells that have been exposed to DRB (*B*). Time of transcriptional inhibition prior to RNA isolation is indicated at the *top* of each lane. β-2-Microglobulin mRNA served as loading control. Result of a nuclear run-on experiment indicates comparable levels of constitutive transcription from IL-2 and GM-CSF genes (*C*). pBR322 was used as negative control.

in vitro transcription data suggested that post-transcriptional mechanisms might account for the observed differences in IL-2 and GM-CSF mRNA accumulation. The rate of decline in mRNA levels in response to treatment with the transcriptional inhibitor, ActD, was examined using poly(A)$^+$ RNA (Fig. 2*A*). GM-CSF mRNA levels decreased rapidly and were undetectable after 30 min of ActD treatment. In contrast, IL-2 mRNA was readily detectable even after 1 h of transcriptional inhibition. In a separate experiment, treatment with the RNA polymerase II-specific inhibitor, DRB, revealed that IL-2 mRNA levels did not significantly decline for up to 16 h of DRB treatment, indicating its property as an unusually stable mRNA in this cell line (Fig. 2*B*).

Detection of Cytoplasmic Proteins with AUUUA Sequence Binding Capability in MLA 144 Cells—Reiterated AUUUA sequences (AU-rich sequences) have been demonstrated to confer, or contribute to, rapid cytoplasmic turnover of cytokine, lymphokine and proto-oncogene mRNAs (6–7), perhaps through the interactions with specific cytoplasmic binding proteins (9–12). The potential role of an altered AUBP-ARE interaction in mediating the observed differential lymphokine mRNA turnover in MLA 144 cells was examined utilizing *in vitro* transcribed, radiolabeled RNA probes containing the 3′-UTR of either human IL-2 (3′-IL-2) or the retrovirally infected gibbon IL-2 (3′-IL-2 PV) as well as an AU-rich region of GM-CSF mRNA (Δ2R1). Cytoplasmic lysates were incubated in the presence of the labeled RNA, followed by UV cross-linking, RNase digestion, and SDS-PAGE. Six major RNA-protein complexes were detected by label transfer. Single bands with molecular masses of approximately 36, 41, 50, 70, and 85 kDa and a double band of 90 kDa were detected (Fig. 3, *lanes 1–3*). Previous studies have established that under these conditions, these proteins are specific for reiterated AUUUA sequences (25). The binding of these proteins (AUBP) to 3′-IL-2 PV occurred at a significantly higher level relative to 3′-IL-2 and to Δ2R1. The observed differential binding between RNA probes was also apparent with lysates from activated human T lymphocytes (Fig. 3, *lanes 6–8*), although cell-dependent differences in overall cytoplasmic AUBP-binding patterns were seen. The dominant AUBP in MLA 144 cytoplasmic lysates were the 90- and 36-kDa complexes, while in activated lymphocyte lysates the 41-, 50-, and the 85-kDa complexes were more prominent than the 36 kDa and equal to that of the 90 kDa (Fig. 3, *lanes 6–8*). Analysis of the detergent-resistant cytoplasmic lysates (INSOL, that include cytoskeleton-associated proteins)

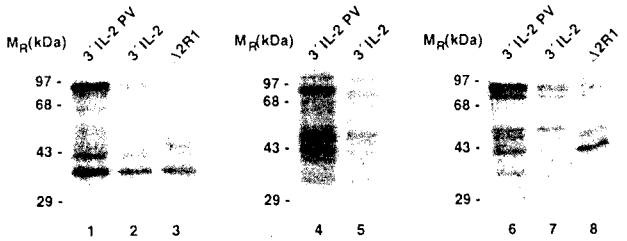
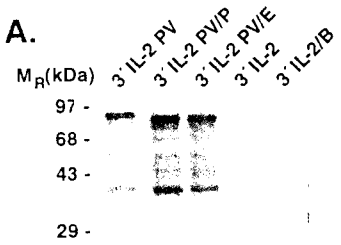


FIG. 3. **Detection of cytoplasmic AUUUA sequence-binding proteins by AUBP assay.** *In vitro* transcribed and radiolabeled RNA probes were incubated with cytoplasmic lysates from MLA 144 cells (*lanes 1–3*) or 8 h of PHA-activated lymphocytes (*lanes 6–8*) or INSOL cytoplasmic lysates from MLA 144 cells (*lanes 4* and *5*). Following UV cross-linking and RNase treatment, the samples were analyzed by SDS-PAGE under denaturing conditions. Radiolabeled probes are indicated at the *top* of each lane.

revealed additional AUBP (32–34 kDa and 100 kDa) with the same differences in binding (Fig. 3, *lanes 4* and *5*).

Effect of Removal of Proviral Sequences from 3′-IL-2 PV on AUBP Binding and Its Affinity—To determine whether the presence of the viral LTR itself specifically influenced AUBP binding to 3′-IL-2 PV distinct from its increase in RNA length, RNA probes were transcribed from templates in which the 3′-IL-2 PV had been truncated using the *Pst*I (3′-IL-2 PV/P) and *Eco*RV (3′-IL-2 PV/E) restriction sites (Fig. 1*B*). 3′-IL-2 PV/P (96 bases) and 3′-IL-2 PV/E (171 bases) generated a similar AUBP pattern to 3′-IL-2 PV, but with an approximately 1.6 or 1.2 times higher intensity (Fig. 4). In contrast, truncation of the human 3′-IL-2 using the *Bgl*I restriction site (3′-IL-2/B) to a comparably sized transcript (126 bases) did not influence AUBP binding relative to 3′-IL-2 (Fig. 4). These data correlated well with relative AUBP affinity for their RNA ligand as demonstrated by cross-competition experiments (Fig. 5). 3′-IL-2 PV/P binding was nearly completely competed by a 50-fold molar excess of the cold 3′-IL-2 PV/P. In contrast, incomplete competition of 3′-IL-2 PV/P binding was evident with a 250-fold molar excess of cold 3′-IL-2 PV (*center left panel*), whereas cold 3′-IL-2 failed to substantially reduce AUBP binding even at this high concentration (*center right panel*). This latter result further confirms the capability of 3′-IL-2 PV to associate with AUBP in a selectively stronger manner relative to 3′-IL-2.

Northwestern Analysis of AUBP Binding to Labeled RNA Probes—During the course of our AUBP experiments, the RNA-protein binding occurs in solution, and may be mediated through a complex of proteins interacting with a specific RNA ligand. Transfer and consequent immobilization of the electrophoresed AUBP of cytoplasmic lysates onto a membrane support enabled investigation whether the differential binding of AUBPs to the various RNA probes was dependent on protein-protein interactions. As demonstrated in Fig. 6*A*, all of the indicated RNA probes bound to proteins of 36 and 43–45 kDa on the membrane support. Binding of each proteins was four times greater to 3′-IL-2 PV relative to 3′-IL-2 (Fig. 6*C*). The binding intensity of 3′-IL-2 PV/E appears to be slightly higher relative to 3′-IL-2 PV. These observations suggest that specific interaction and differential binding of these RNA probes to AUBP occur independently of protein-protein interactions. In contrast, comparison of the binding of 3′-IL-2 *versus* 3′-IL-2/B yielded different results than that observed with AUBP assay, *i.e.* 3′-IL-2/B seemed to bind approximately two times more intensely than 3′-IL-2 to the membrane-bound AUBP. Subsequent Northwestern analyses, however, did not show enhanced AUBP binding to 3′-IL-2/B relative to 3′-IL-2, whereas the increased binding of 3′-IL-2 PV and 3′-IL-2 PV/E was consistently observed (data not shown). Previous work demonstrated



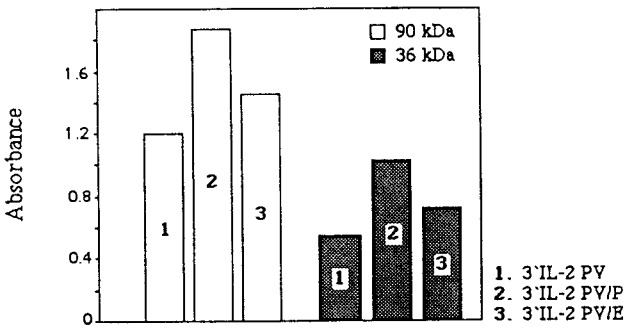


FIG. 4. **Effect of removal of viral LTR sequences from the 3′-IL-2 probe on the level of AUBP binding.** *A*, cytoplasmic lysates were incubated in the presence of each of the indicated radiolabeled RNA probes, UV cross-linked, RNase treated, and analyzed by SDS-PAGE. 3′-IL-2 PV/P, PV/E and 3′-IL-2/B probes were transcribed from DNA templates generated as indicated in Fig. 1*A*. Binding intensity of the 36- and 90-kDa proteins were evaluated by quantitative densitometric analysis (*B*).

that two AUBP found in cytoplasmic lysates of activated human T lymphocytes were members of the hnRNP family (25). A 36-kDa protein (hnRNP A1) and a 43-kDa protein (member of the hnRNP C protein family) were identified as capable of specific binding to reiterated AUUUA motifs. In order to ascertain whether the hnRNP A1 and C proteins can be among the complexes formed with the labeled RNA probes, we analyzed the membranes by immunoblotting. As shown in Fig. 6*B*, the band detected with antibody to hnRNP C appears to comigrate with the 43–45-kDa complex detected by Northwestern analysis. Additionally, the higher molecular mass isoform of hnRNP A1 appears colocalized with the 36-kDa size band on the Northwestern blot. Given the knowledge that hnRNP A1 undergoes considerable post-translational modification (reviewed in Ref. 26), the presence of two immunoreactive proteins suggests isoforms of the same gene product, with different capacity to interact with reiterated AUUUA sequences when immobilized. Alternatively, the doublet may represent highly homologous or nearly identical forms of hnRNP A1 generated from closely related genes or differential processing or translation of the hnRNP A1 mRNA (27, 28).

Differential Binding of Recombinant A1, C1, and La Proteins to in Vitro Transcribed RNA Probes—In order to correlate the immunoblotting and Northwestern data as well as determine the specificity of the observed increase in RNA binding of provirally modified IL-2 3′-UTR with known RNA-binding proteins in solution, the binding of recombinant human hnRNP A1, C1, and La proteins to labeled RNA probes in AUBP assays was examined. The autoantigen SS-B/La, a 47-kDa protein, was chosen because it has been shown to bind 3′-terminal oligouridine residues found in RNA polymerase III transcripts (29).

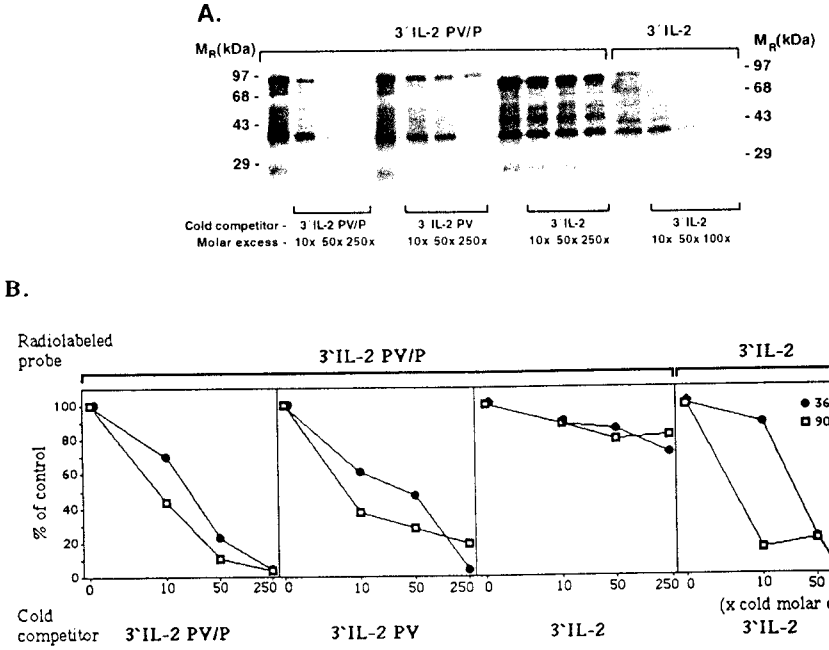


FIG. 5. **Analysis of AUBP binding affinity of *in vitro* transcribed RNA probes derived from virally altered and native IL-2 3'-UTRs.** Competition experiments were performed with ^{32}P-labeled and cold RNA transcripts added simultaneously in the binding reaction together with cytoplasmic lysates from MLA 144 cells (*A*). Labeled RNA probes are shown *above* the lanes, while cold competitors together with molar excesses of the cold probes are indicated at the *bottom*. Diagrams conclude the data from the densitometric analysis of the binding of the 36- and 90-kDa proteins from the same experiments (*B*).

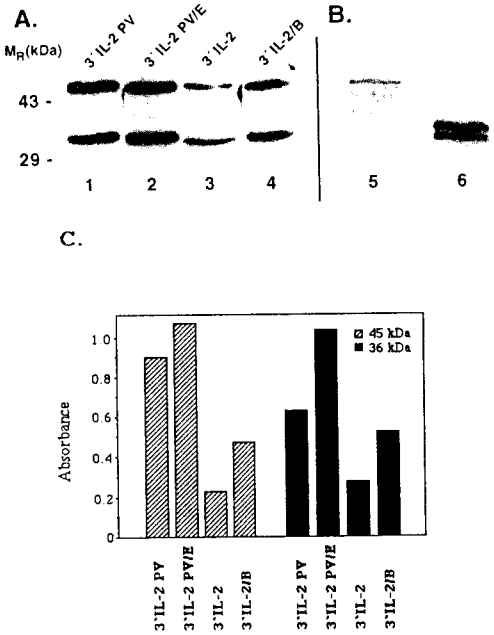


FIG. 6. **Northwestern analysis of AUBP binding to radiolabeled probes.** *A*, AUBPs were immobilized on membrane support prior to interaction with probe RNAs (indicated at the *top* of each lane) at 5 × 10^5 cpm/ml binding solution. The same membranes that are shown in *lanes 2* and *4* were examined by immunoblotting utilizing antibodies 4F4 and 4B10 directed against hnRNP C (*lane 5*) and A1 (*lane 6*), respectively (*B*). Intensity of binding in the Northwestern experiment was quantitated by densitometric analysis (*C*).

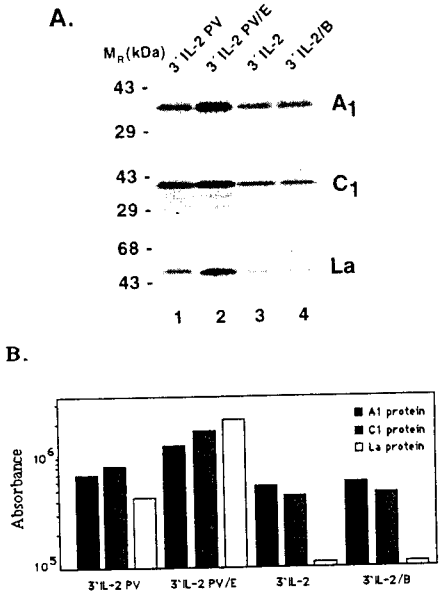


FIG. 7. **Recombinant hnRNP A1, C1, and recombinant La proteins were analyzed in *in vitro* AUBP assay for their ability of binding to the indicated RNA probes.** *A*, 0.25 μg of A1 and La and 50 ng of C1 proteins were incubated in the presence of 8 × 10^4 cpm probes, and following UV cross-linking and RNase treatment, samples were analyzed on SDS-PAGE under reducing conditions. Quantitative evaluation of binding differences of each of these proteins to the RNA ligands was made by densitometric measurements, with longer exposures used for La, relative to hnRNP A1 and C1 (*B*).

Both recombinant hnRNP A1 and C1 proteins demonstrated a hierarchy of RNA binding identical to that observed by both AUBP assay and Northwestern blotting (Fig. 7). In contrast, although recombinant La yielded a comparable binding profile of 3'-IL-2 PV and 3'-IL-2 PV/E (Fig. 7), very little binding to 3'-IL-2 or 3'-IL-2/B was observed, perhaps indicating recognition of an RNA sequence distinct from that bound by hnRNP A1 or C1.

DISCUSSION

The MLA 144 T cell line has been known to constitutively produce cytokines such as IL-2 and GM-CSF (18). In this report, we demonstrate that MLA 144 cells selectively accumulate at least a >10-fold greater level of IL-2 mRNA relative to GM-CSF despite comparable rates of gene transcription, suggesting that considerable differences in the regulation of their respective mRNA turnover exist. The rate of IL-2 mRNA turnover was found to be markedly prolonged relative to that of

GM-CSF mRNA, which was rapidly degraded with kinetics similar to those reported by others (6, 30, 31). These findings indicated differential post-transcriptional regulation of lymphokine mRNA, both of which contained reiterated AUUUA motifs (ARE) in their 3′-UTR. Thus, these effects might have been mediated by altered expression of AUBP that can differentially bind to the 3′-UTR of IL-2 and GM-CSF. Alternatively, the presence of a 495-base long proviral insertion in the IL-2 mRNA might have altered the ability of the AUBP to bind to the ARE present in the 3′-UTR. Correlation of this modification of the 3′-UTR of IL-2 with AUBP binding *in vitro* was of considerable interest because the retroviral insertion resulted in loss of the 3′ 180 nucleotides including three AUUUA motifs, while preserving four nearly continuous reiterations of AUUUA with one AUUUUA sequence, each of which have been shown to bind similar proteins (4). This sequence is very similar to the reiterated ARE within the GM-CSF 3′-UTR, which has been demonstrated to confer instability in heterologous gene constructs (6).

To address these issues, we examined the ability of AUBP to bind radiolabeled RNA probes containing the 3′-UTR of native and provirally modified IL-2 as well as the reiterated ARE of GM-CSF in different cell types. Comparable AUBP patterns were demonstrated in the detergent-soluble and -insoluble cytoplasmic fractions of MLA cells as well as of PHA-activated human lymphocytes, indicating no major qualitative differences in AUBP binding or their subcellular localization in MLA 144 cells relative to those (normal human T) cells in which IL-2 mRNA is labile. These data suggest that expression of a novel AUBP in MLA 144 cells cannot account for the altered IL-2 mRNA turnover. Although quantitative differences in specific RNA·AUBP interactions in different cell types were observed and cannot be definitively excluded as possible factors, it seems unlikely to account for the marked change in IL-2 mRNA turnover observed in MLA 144 cells. More striking was the finding that independent of cell type, the ARE of 3′-IL-2 PV bound AUBP with higher intensity compared to those of 3′-IL-2 or Δ2R1, suggesting that AUBP binding *in vitro* directly correlated with the abnormal IL-2 mRNA stability. Therefore, AUBP binding was not dependent on the absolute number of AUUUA pentamers of the RNA ligands: 3′-IL-2 PV with four motifs bound AUBP with higher intensity than 3′-IL-2 with seven. Additionally, the number of continuous AUUUA pentamers did not correlate with increased binding either (Δ2R1 has four continuously aligned pentamers). These findings were further supported by truncation experiments in which removal of the distal three AUUUA elements from the 3′-IL-2 probe (3′-IL-2/B) did not appear to affect AUBP binding.

In contrast to truncation of native IL-2 3′-UTR, shortening of the provirally modified IL-2 3′-UTR yielded enhanced AUBP binding affinity in cross-competition experiments. These experiments indicated that the increased affinity of AUBP binding of the IL-2 PV probe appeared to be mediated within the first 20 bases of the proviral insertion. The remaining approximately 500 bases (which contained the majority of proviral insertion) appeared to play little role in the increased AUBP binding of 3′-IL-2 PV relative to 3′-IL-2. As stated above, the effects of truncation appeared to be specific for 3′-IL-2 PV, as comparable binding of 3′-IL-2 and 3′-IL-2/B were observed. Thus, altered AUBP binding was unaffected by random shortening or lengthening of RNA probes, which was consistent with a previous report (4). Nevertheless, the enhanced ability of AUBP to bind 3′-IL-2 PV indicates something distinct about the modification of the 3′-IL-2 UTR by the proviral insertion. Because this finding correlates with increased IL-2 mRNA stability, it is therefore of considerable functional interest in understanding ARE-dependent mRNA turnover. Our data further indicate that either the addition of the first 20 nucleotides (contained within 3′-IL-2 PV/P) or the deletion of the four nucleotides (GTAA) at the site of proviral insertion (20) altered the secondary structure of the ARE-RNA ligand in such a way to enhance binding by AUBP. These data would therefore suggest that the secondary structure and context of a particular ARE sequence can modify AUBP-binding and ARE-dependent mRNA turnover. Finally, it raises concerns about the ability to examine the effects of ARE on mRNA turnover and translation in heterologous constructs, where the context and secondary structure may be considerably different from what is present in the 3′-UTR of cytokine/proto-oncogene mRNA.

In previous work, hnRNP A1 and C have been shown to be AUBP found in cytoplasmic lysates from human T lymphocytes (25). Northwestern blotting demonstrated that ARE-AUBP binding paralleled that seen in solution. This technique also permitted colocalization of hnRNP A1 and C proteins with the 36- and 43-kDa AUBP, respectively, by Western blotting. These studies prompted demonstration that recombinant hnRNP A1 and C1 proteins exhibited a similar hierarchy of binding as did the AUBP detected by cross-linking or Northwestern analysis, indicating their probable identity. The La/SS-B protein, which has been shown to bind oligouridylate sequences (29), was markedly less able to bind the ARE in native 3′-IL-2 or 3′-IL-2B than hnRNP A1 and C. Thus, the uridine-rich stretches recognized by the La/SS-B antigen appear to be differentially accessible for binding in 3′-IL-2 PV and 3′-IL-2, relative to the ARE-containing RNA ligand recognized by hnRNP A1 and C. These data provide further evidence, independent of ARE binding, that the secondary structure of the 3′-UTR of IL-2 has been substantially altered by the proviral insertion.

The observed correlation between AUBP binding and IL-2 mRNA turnover in MLA cells appears different from the finding of a cytoplasmic AU-rich element-binding factor of an erythroleukemia cell line which exhibited destabilizing activity in the analysis of *c*-myc mRNA in a cell-free mRNA decay system (12). Additionally, Bohjanen *et al.* (9) reported a 30-kDa AUBP protein whose disappearance correlated with increased lymphokine mRNA stability. It is therefore likely that there are several AUBP that might conceivably bind to a specific ARE, which may either stabilize or destabilize a specific mRNA or influence its translation. Our data was not designed to conclusively identify the AUBP which alters IL-2 mRNA stability *in vivo*. Rather, these data suggest that modification of the primary sequence in a very small region of the IL-2 3′-UTR enhances mRNA stability *in vivo* as well as its ability to be bound by several different AUBP (both known and unknown). In this regard, it should be noted that two of the proteins in cytoplasmic lysates (hnRNP A1 and C), whose increased binding to the IL-2 ARE correlated with the proviral insertion, have been implicated with the regulation of pre-mRNA processing (25). Thus, these proteins appear to have an established role in RNA metabolism that may vary as a consequence of intracellular location. Furthermore, with the recognition that hnRNP A1 can shuttle between the nucleus and cytoplasm (32), our correlation of its binding with altered mRNA stability suggests an additional role in cytoplasmic mRNA turnover.

In conclusion, we propose that the combination of a low level of constitutive transcription of an abnormally stable mRNA encoding IL-2 in the MLA 144 cell line (both events retrovirally mediated) played a major role in malignant transformation of a T cell. Production of abundant levels of IL-2 induced autocrine growth stimulation, thereby permitting eventual growth factor-independent cell growth and tumorigenesis. A similar oncogenic role of altered mRNA turnover is suggested by the finding of increased stability of proto-oncogene and cytokine mRNA in several tumors (15–17). Our results additionally offer a direct

correlation between the intensity and affinity of specific AUBP binding with mRNA stability. These studies highlight the complexity of examining both the interaction of *trans*-acting proteins with sequence-specific RNA ligands independent of their natural context. The corollary of this observation is that studies of putative destabilizing sequences in chimeric gene constructs may be limited in application due to the confounding influence of altered secondary structure. Finally, although our studies demonstrate a correlation between ARE-dependent mRNA turnover *in vivo* and AUBP binding *in vitro* in the differential regulation of two cytokine mRNA stability, they do not establish causality between these observations. The comparable patterns and intensity of AUBP binding of the native and provirally modified 3′-IL-2 UTR in normal human T cells (in which IL-2 and GM-CSF are each unstable) would seem to support the view that these effects are mediated in an ARE-dependent manner. Comparison of the normal and provirally modified IL-2 allele mRNA turnover in the MLA cell would help resolve this question. Unfortunately, induction of transcription of the non-virally modified allele has been unsuccessful. Therefore, future studies will be directed at identifying the nature of the proviral sequence which appears to modify AUBP binding. As a result, we hope to gain new insights into both retroviral induction of the malignant T cell phenotype as well as mechanisms of eukaryotic mRNA turnover regulation.

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COMBINED APPLICATION OF *IN VIVO* UV-CROSSLINKING AND *IN VITRO* LABEL TRANSFER IN THE EXAMINATION OF AU-RICH SEQUENCE BINDING PROTEIN - RNA INTERACTIONS.

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Abbreviations: ARE (AU-rich element), AUBP (AU-rich sequence element binding proteins), 3′ UTR (3′ untranslated region), IL-2 (interleukin-2), FBS (fetal bovine serum), PHA (phytohemagglutinin), PMA (phorbol myristyl acetate), Iono (Ionomycin), DTT (dithiothreitol), SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), CAPS (3-[cyclohexamino 1-propanesulfonic acid]), hnRNP A1 (heterogeneous nuclear ribonucleoprotein A1), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), RER (rough endoplasmic reticulum).

ABSTRACT

*A combination of **in vivo** UV light-induced crosslinking of nucleic acids to proteins and **in vitro** label transfer assay was applied to investigate specific interactions between AU-rich sequences (ARE) in the 3′ UTR of lymphokine mRNAs and cytoplasmic AU-rich sequence element binding proteins (AUBP) in normal human lymphoblasts and MLA 144 gibbon lymphoid tumor cells. We demonstrate that a pool of cytoplasmic AUBP can be effectively crosslinked to RNA **in vivo**, suggesting a close association of these proteins with ARE sequences in the cytoplasm. We also show that the UV-crosslinked AUBP pool is markedly reduced in malignantly transformed MLA 144 cells compared with normal lymphoblasts, indicating weaker interaction between lymphokine ARE and AUBP in these tumor cells. Similar differences in AUBP-RNA associations were found between the membrane-bound polysomal subfractions of the two cell types where most of the AUBP activity was localized. We suggest that the decreased AUBP-mRNA association in MLA 144 cells might reflect a process concerned with disturbances of mRNA metabolism in the neoplastic phenotype.*

INTRODUCTION

Selective regulation of cytoplasmic mRNA turnover is an important control mechanism in the expression of a variety of eukaryotic genes (Ross, 1988; Atwater et al., 1990; Belasco and Brawerman, 1993). This mechanism has been shown to contribute to rapid and precise lymphokine, cytokine and proto-oncogene production in response to various environmental stimuli, such as cell activation. Many of these factors are encoded by shortlived mRNAs with a unique 3′ UTR containing reiterations of the motif AUUUA in a AU-rich context. The presence of AU-rich elements (ARE) has been associated with intrinsic cytoplasmic instability of a number of different mRNAs (Shaw and Kamen, 1986; Peppel et al., 1991). Although the exact mechanism by which ARE reduce the turnover rate of mRNAs is not yet clear, the identification of cytoplasmic AU-sequence binding proteins (AUBP) and the capability of AUBP to specifically bind to ARE sequences *in vitro*, emphasize the potential role of AUBP in selective mRNA catabolism (Malter, 1989; Bohjanen et al., 1991; Vakaloupoulou et al., 1991). Previous work in our laboratory has described cytoplasmic AUBP with specific lymphokine ARE-binding capability in activated normal human

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lymphocytes (Hamilton et al., 1993) and MLA 144 tumor cells (Henics et al., 1994). Among these, two 36-kDa proteins have been identified as heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) and glyceraldehyde-3-phosphate dehydro- genase (GAPDH) and their *in vitro* RNA binding activities have been characterized (Hamilton et al., 1993; Nagy and Rigby, 1995). Intriguingly, higher *in vitro* AUBP binding affinity has been correlated with substantially increased stability of IL-2 mRNA in MLA 144 cells (Henics et al., 1994). Moreover, phorbol ester or calcium ionophore induced transient stabilization of lymphokine mRNAs might be mediated by AUBP binding to the 3′ ARE of the mRNA (Malter and Hong, 1991). Thus, selective control of the interactions between mRNA and various AUBP might be a critical mechanism which regulates the cytoplasmic turnover of labile mRNAs.

In an attempt to examine specific mRNA-AUBP interactions in lymphoid cells *in vivo*, we have combined the techniques of UV-crosslinking with subsequent label transfer assay. The exposure of cells to UV light allows the preservation of mRNA-AUBP complexes which exist *in vivo* at the time of UV irradiation. Subsequent analysis of these complexes by *in vitro* label transfer assay, using a radiolabeled AU-rich RNA probe, provides the detection of non-crosslinked AUBP as an indirect measure of the portion of AUBP complexed *in vivo* with ARE. We demonstrate that AUBP from normal human lymphoblasts and MLA 144 lymphoid tumor cells can be effectively crosslinked to RNA molecules *in vivo*. Our data also indicate differences in the association of cytoplasmic AUBP with RNA in the normal and neoplastic cytoplasm. We also show that cytoplasmic AUBP localizes predominantly in the membrane-bound polysomal compartment of lymphoid cells. Finally, we suggest that the combination of UV light-induced *in vivo* crosslinking and *in vitro* label transfer assay is a valuable means of studying sequence specific RNA binding protein-RNA interactions *in vivo*.

MATERIALS AND METHODS

Reagents: PHA was purchased from Wellcome Reagent Ltd. (Beckenham, England). PMA was from Sigma Chemical Co. (St. Louis, MO), and Ionomycin from Calbiochem (San Diego, CA). Recombinant human IL-2 was generously provided by Cetus Corporation (Emeryville, CA). [^{32}P]-UTP (~3000 Ci mmole^{-1}) was obtained from Amersham (Arlington Hts., IL), unlabeled nucleotides were purchased from Boehringer-Mannheim (Indianapolis, IN). 4B10 monoclonal antibody against hnRNP A1 was generously provided by Dr. Gideon Dreyfuss and the 40.10.09. Anti-GAPDH antibody was a gift of Dr. Michael Sirover.

Cell culture and activation: MLA 144 gibbon lymphoid tumor cell line (ATCC, Rockville, MD) was maintained in RPMI 1640 medium (JRH Biosciences, Lenexa, KS) supplemented with 8% heat inactivated (30 min, 56°C) FBS (Flow Laboratories, McClean, VA) at a density of 0.5-1x10^6 cells/ml. Peripheral venous blood samples of healthy volunteers were separated individually on discontinuous Ficoll-Hypaque gradients and the lymphocytes were cultured in 8% FBS supplemented RPMI 1640 medium at a density of 0.5-1x10^6 cells/ml. Lymphoblasts were generated by subsequent maintenance of PHA activated (1 g/ml, 3 days) lymphocytes of individual donors in the presence of 25 U/ ml recombinant IL-2 in 8% FBS supplemented medium at a density of 10^6 cells/ml (referred to as IL-2 lymphoblasts). IL-2 lymphoblasts and MLA 144 cells were cultured in the absence or presence of 10 nM PMA and 1 M Ionomycin for 0, 4 or 20 h.

***In vivo* UV-crosslinking and preparation of cytoplasmic lysates:** Following activation, cells were pelleted, resuspended in cold PBS, distributed into 6-well tissue culture plates (1.6x 10^7 cells/well) and exposed to 254 nm UV light on ice in UV Stratalinker 1800 (Stratagene, La Jolla, CA), (3000 Wcm^{-2}) for 0, 2 and 8 or 0, 2.5 and 8.5 min. Cells were then harvested and lysates were prepared as described (Cervera et al., 1981). Briefly, cells were washed in ice-cold PBS, pellets were resuspended in a buffer containing 10 mM PIPES, pH 6.8, 100 mM KCl, 2.5 mM $MgCl_2$, 300 mM sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100 and incubated on ice for 3 min. Following centrifugation for 3 min at 900xg, supernatants were collected, aliquoted and immediately frozen. To ascertain that the UV irradiation was specific only for

the fixation of close nucleic acid-protein complexes and that this procedure did not affect the extractability of proteins in the various lysate fractions, total protein concentrations (BCA Microprotein Kit, Pierce) of these cytoplasmic fractions were compared from control and UV-irradiated cells. There were no differences in the yields of total proteins extracted (data not shown), as would otherwise be expected if non-specific protein-protein crosslinking was formed.

RNA probe and *in vitro* label transfer (AUBP) assay: A 97 base ^{32}P-labeled probe (*PV-PstI*) was transcribed with T3 polymerase from a DNA template that had been generated by *BglI* and subsequent *PstI* digestion of the plasmid pT7/T3 19 containing the 3′ UTR of the IL-2 mRNA from MLA 144 cells (Henics et al., 1994). Probe molecules with specific activity > 10^8 cpm g^{-1} RNA were generated by using 50 Ci [^{32}P]-UTP (~3000 Ci mmole^{-1}), 0.0125 mM UTP and 2.5 mM e.a. ATP, CTP and GTP in the transcription reaction (Figure 1A). 8x 10^4 cpm of probe RNAs (~10 fmoles) were incubated with 5 g total protein from cytoplasmic lysates of polysomal preparations in 12 mM HEPES pH 7.9, 15 mM KCl, 0.2 M DTT, 0.2 g ml^{-1} yeast tRNA and 10% glycerol for 10 min at 30°C. RNA-protein complexes were then UV-crosslinked on ice (5 min, 3000 Wcm^{-2}), followed by digestion with RNase T1 (10 U) and A (20 g) for 15 min at 37°C. Samples were then separated by 12.5% SDS-PAGE, gels were dried and analyzed by autoradiography.

Western blotting: 20 g total proteins of crude cytoplasmic lysates or polysomal fractions were separeted by 12.5% SDS-PAGE. Gels were equilibrated in transfer buffer (10 mM CAPS pH 11.0, 15 % methanol) and transferred at 35 V onto nitrocellulose membrane (Hybond-C, Amersham). Blocking and all incubations were performed in TBS-T buffer (20 mM Tris-HCl pH 7.6, 140 mM NaCl, 0.05% Tween-20). For detection, the ECL Chemiluminescent Kit (Amersham) was used.

Preparation of polysomal subfractions: 10^8 IL-2 lymphoblasts or MLA 144 cells were placed in glass Petri dishes (d=20 cm) and exposed to UV light for 0, 2.5 or 8.5 min as described, under continuous gentle agitation. Cells were collected and incubated in 1.5 ml ice-cold PB-I (Polysomal Buffer I) (10 mM Tris-HCl pH 7.6, 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.05% Triton X-100, 100 g/ml Heparin and 0.5 M Pefabloc) for 10 min followed by centrifugation at 200x g for 5 min (Hesketh et al., 1994). Supernatants were saved on ice (Sup I), pellets were washed once in the same buffer, then incubated in 1.5 ml PB-II (same as PB-I except with 130 mM KCl) for 10 min on ice and centrifuged at 800x g for 10 min. Supernatants were saved on ice (Sup II) and pellets were resuspended in ice-cold PB-III (same as PB-I except with 0.5% Triton X-100 and 0.5% Sodium-deoxycholate), lysed for 10 min and centrifuged at 1850x g for 10 min. Supernatants were stored on ice (Sup III) and pellets were discarded. Sup I-III were overlayered on 15 ml 40% sucrose cushions and centrifuged at 32 000x g at 4°C for 16 h. Polysomal pellets from Sup I, II and III were resuspended in a buffer containing 12 mM HEPES pH 7.9, 45 mM KCl and frozen immediately as "free" (FPS), cytoskeleton-associated (CYPS) and membrane-bound (MBPS) polysomal subfractions, respectively (Hesketh et al., 1994). Protein contents in each fraction were determined by the BCA Microprotein Kit (Pierce).

RESULTS

To examine the relationship of specific AUBP with the 3′ ARE of labile lymphokine mRNAs *in vivo*, we exposed normal human lymphoblasts and MLA 144 tumor cells to UV light *in vivo* prior to preparation of cytoplasmic lysates. AUBP activity was assayed using a radiolabeled RNA probe that contained 4 AUUUA pentamers (Figure 1A). The IL-2 *PV-PstI* probe was chosen since it is bound by cytoplasmic AUBP with high affinity (Henics et al., 1994). With this technique, we detected at least six cytoplasmic AUBP out of which the 90-, 50- and the 43-kDa proteins were labeled most intensely with the probe, but other proteins, including a 36-kDa one, also appeared (Figure 2). Increasing the time of UV exposure from 0 to 2, or from 2 to 8 min resulted in the reduction of the 90-, 50- and 43-kDa AUBP binding activity in both cell types (Figure 2). Substantial differences in the extent of the decline in AUBP activity was observed in normal and tumor cells. In normal IL-2 lymphoblasts, after 8 min of *in vivo* UV exposure,

A. 5`-GGGA-n_{10}-CU**AUUUAUUUA**AAU**AUUUA**AAUUUUAU**AUUUA**UUG-n_{30}-
-UAUUAUUCUUAAAGAGC-3`

B.

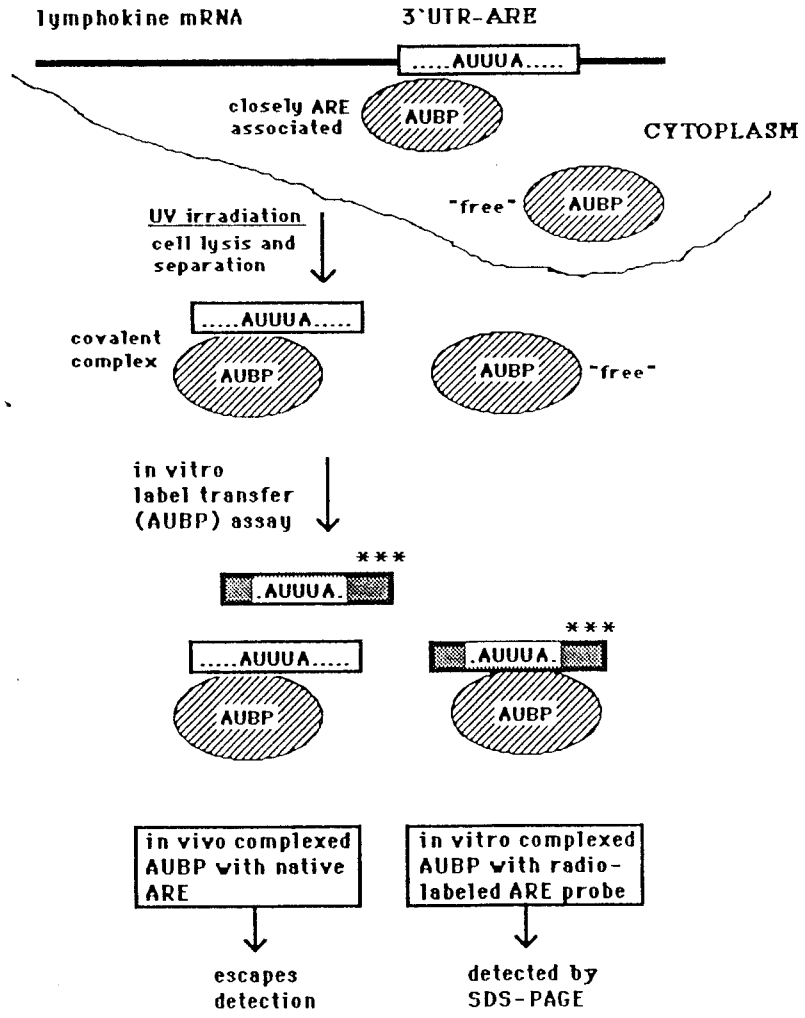

Figure 1. A) Sequence of *in vitro* transcribed *PV-PstI* RNA probe with the AUUUA motifs underlined. **B)** Combination of *in vivo* UV-crosslinking and *in vitro* label transfer techniques in the analysis of intracellular associations of lymphokine mRNA and specific 3′ UTR-ARE binding proteins (AUBP). Assuming a regulated balance in the distribution of ARE-associated and "free" AUBP, exposition of living cells to UV light renders ARE-associated AUBP to be covalently crosslinked to RNA. Subsequent detergent mobilization of ARE-AUBP complexes and "free" AUBP enables detection only of free AUBP by means of *in vitro* complex formation with radiolabeled probes containing specific lymphokine ARE.

AUBP activity of the 90-kDa protein was reduced by ~ 90%, whereas the 50-kDa and 43-kDa activity declined by ~50% and ~40% after 8 min, respectively (Figure 3, panel A, B and C). In contrast, analysis of lysates obtained from unactivated MLA cells showed that the 90- and 50-kDa proteins were reduced by only ~25 and ~30%, respectively (Figure 3, panel A, B). The *in vitro* detectable 43-kDa AUBP activity was reduced by ~50% after 8 min of UV exposure, comparable to that seen in IL-2 lymphoblasts (Figure 3, panel C). In general, UV-crosslinkable AUBP pools from MLA cells were always smaller than those from IL-2 lymphoblasts in multiple experiments.

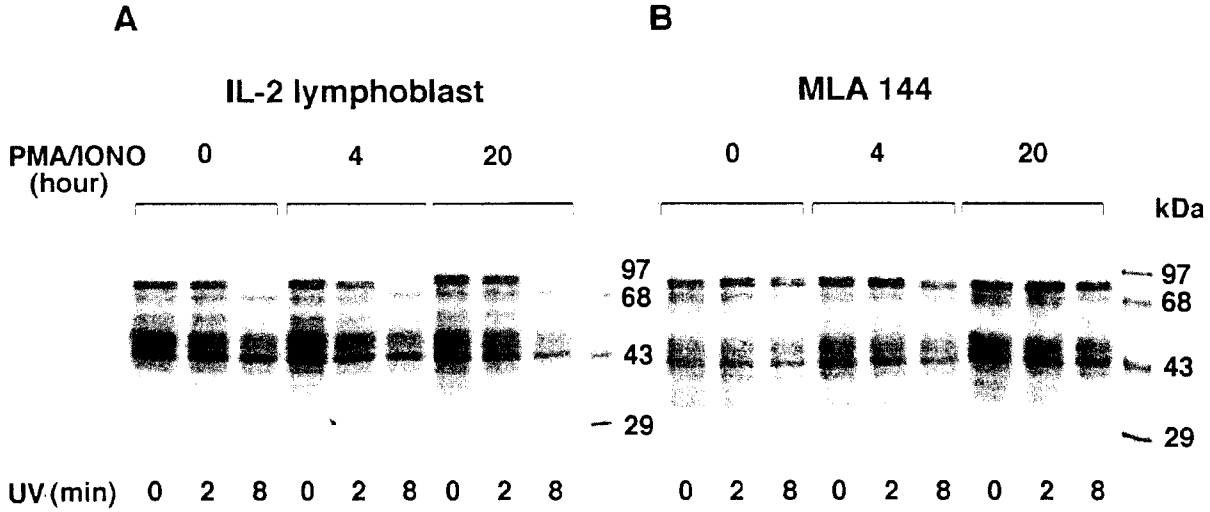

Figure 2. **A)** Autoradiographic detection of specific cytoplasmic AUBP from normal human IL-2 lymphoblasts. Cells were activated with PMA and Ionomycin for 0 (left panel), 4 (middle panel) or 20h (right panel) prior to *in vivo* UV-crosslinking for 0, 2 and 8 min. **B)** Autoradiogram of specific cytoplasmic AUBP from untreated (left panel) or 4 (middle panel) and 20h (right panel) PMA-Ionomycin activated MLA 144 cells. Detergent-extracted cytoplasmic lysates were incubated with radiolabeled probe RNA containing an MLA IL-2 ARE fragment (*PV-PstI*) with 4 AUUUA pentamers, and exposed to UV light and RNase treatment. Samples were then analyzed by SDS-PAGE under reducing conditions.

As has been proposed by Malter and Hong (1991), molecular mechanisms involved in phorbol ester- and calcium ionophore-induced lymphokine mRNA stabilization may be mediated through AU-rich element binding proteins. To address the question of whether altered ARE-AUBP associations *in vivo* could be detected by our combined method, we UV-crosslinked activated cells and monitored their extracted ARE-AUBP complexes. Analysis of lysates from PMA/ Ionomycin-activated cells revealed that, in IL-2 lymphoblasts, the 90-kDa UV-crosslinkable AUBP was unaffected by activation, whereas the same pool of the 50- and 43-kDa AUBP increased (Figure 2, Figure 3, panel A, B and C). In activated MLA cells, similarly to IL-2 lymphoblasts, the behaviour of the 90-kDa AUBP upon UV exposure appeared to be unchanged by activation. Interestingly, while the UV-crosslinkable 50-kDa AUBP was more apparent following activation, the 43-kDa AUBP declined by the end of the 20 h PMA/Ionomycin treatment (Figure 2, Figure 3, panel A, B and C).

In order to demonstrate that the absolute level of extracted RNA binding proteins was unchanged by UV crosslinking, we measured the amount of two known AUBPs, hnRNP A1 and GAPDH in the same cytoplasmic lysates as a function of UV exposure (Figure 4). The AUBP activity of these proteins has been described previously and characterized in lymphoid cells (Hamilton et al., 1993; Nagy and Rigby, 1995). *In vivo* UV exposure did not affect the

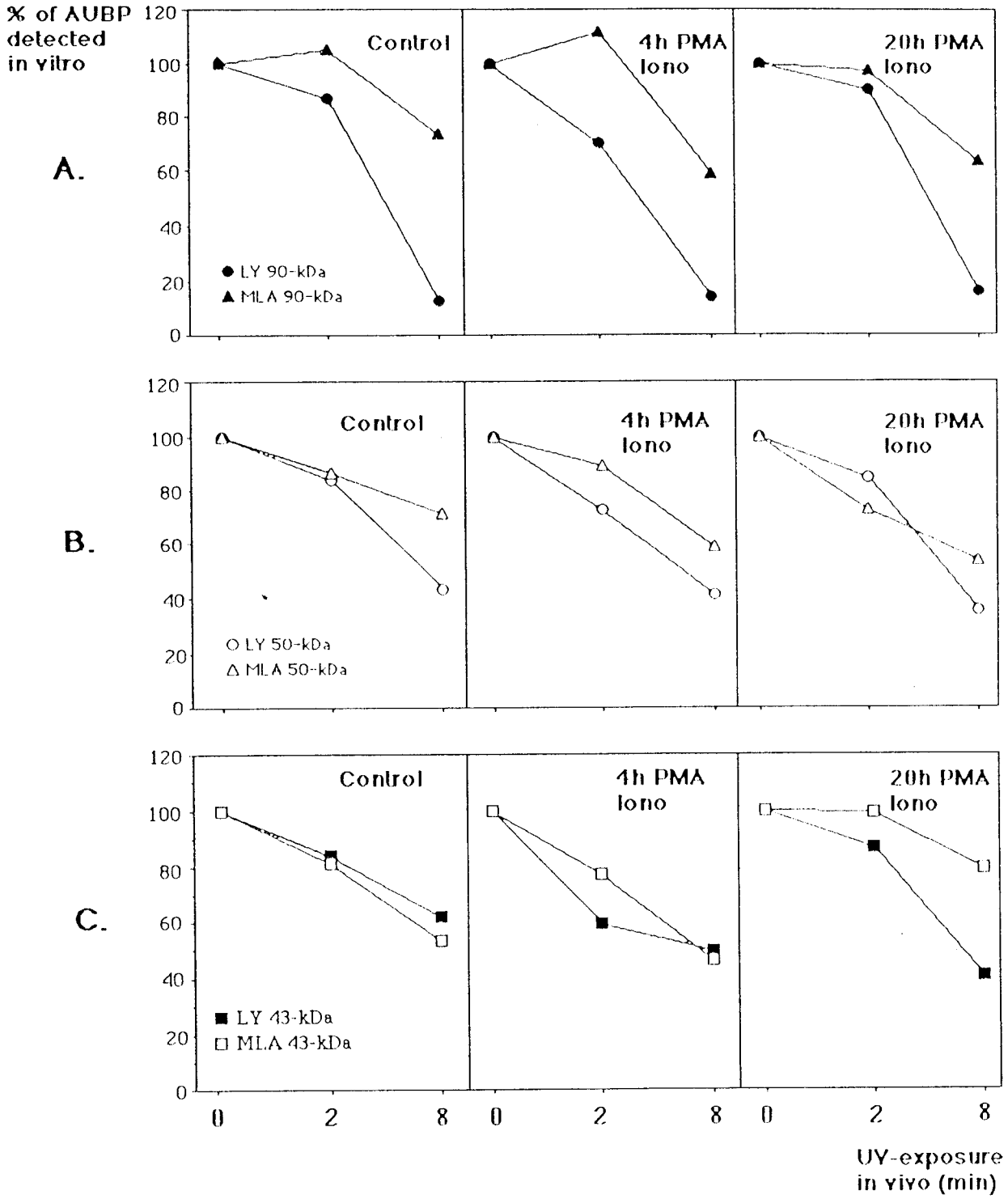

Figure 3. Changes in the detectable levels of cytoplasmic AUBP after various times of *in vivo* UV light exposure of normal human IL-2 lymphoblasts and MLA 144 lymphoid tumor cells. The indicated Mw complexes (90-, 50- and 43-kDa in panel A, B and C, respectively) were quantitated by densitometric analysis of the autoradiograms shown in Figure 2 and the relative values at various times of *in vivo* UV irradiation were expressed as % of the control (0 min UV exposure). Duration of PMA-Ionomycin activation is indicated within the boxes. Time of *in vivo* UV light exposure is shown at the bottom of the figure.

amount of hnRNP A1 and GAPDH in the lysates, indicating that a decrease in their levels could not account for the decreased AUBP activity *in vitro*. Rather, these data suggest that UV light covalently fixes AUBP in their complexes with RNA *in vivo*, thereby blocking their ability to bind radiolabeled RNA *in vitro*.

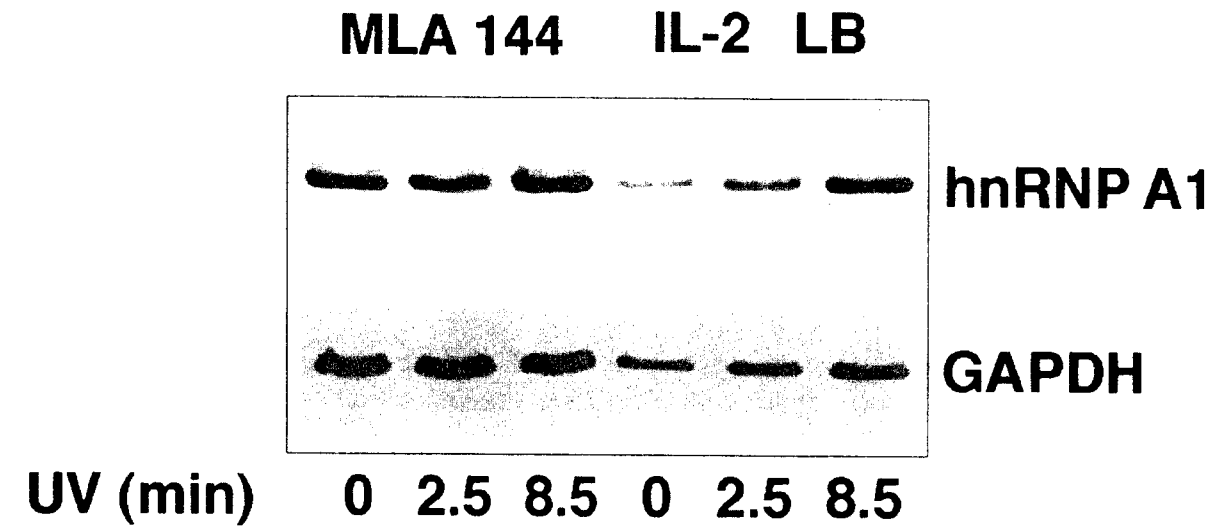

Figure 4. Western analysis of cytoplasmic AUBP, hnRNP A1 and GAPDH in IL-2 lymphoblasts and MLA 144 cells. Cells were UV irradiated for various times, cytoplasmic lysates were obtained and separated by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes and immuncomplexes were visualized by chemiluminescence. Duration of *in vivo* UV exposure prior to cytoplasmic lysate preparation is indicated under each lanes.

In an attempt to gain insight into the subcellular localization of AUBP activity in general, and to determine if AUBP associate with polysomes in particular, we prepared three polysomal sub-fractions of control as well as UV-exposed IL-2 lymphoblasts and MLA cells. "Free", cytoskeleton-associated and membrane-bound polysomal subfractions (FPS, CYPS and MBPS, respectively) were prepared by a sequential salt-detergent lysis and extraction. These fractions were subjected to AUBP assay and Western blotting (Figure 5). The majority of AUBP activity was detected in the MBPS in both IL-2 lymphoblasts and MLA cells (Figure 5A). FPS and CYPS fractions possessed considerably less AUBP activities despite their comparable RNA content by OD_{260} (not shown). Interestingly, in the MBPS, optimal UV- crosslinking and label transfer occured with the 36- and 43-kDa AUBP, while the larger complexes were less evident (Figure 5A). When MBPS were analyzed after *in vivo* UV exposure, the overall detected AUBP activity decreased in these fractions of both cell types (Figure 5A). The decline in the 36-kDa AUBP was larger in IL-2 lymphoblasts relative to MLA cells (Figure 5B). To ascertain that the observed UV-dependent decrease in *in vitro* AUBP activity was not due to diminished protein levels in the fractions, we analyzed each of the preparations by immunoblotting for a known 36-kDa AUBP (Figure 5C). This experiment revealed that the level of the previously characterized AUBP, hnRNP A1 (Hamilton et al., 1993) did not change by UV exposure in the MBPS of the two cell types.

DISCUSSION

The mechanism by which cytoplasmic AU-rich RNA binding proteins, found in many eukaryotic cells, contribute to the turnover regulation of labile mRNAs is unclear (Sachs, 1993). One difficulty in the

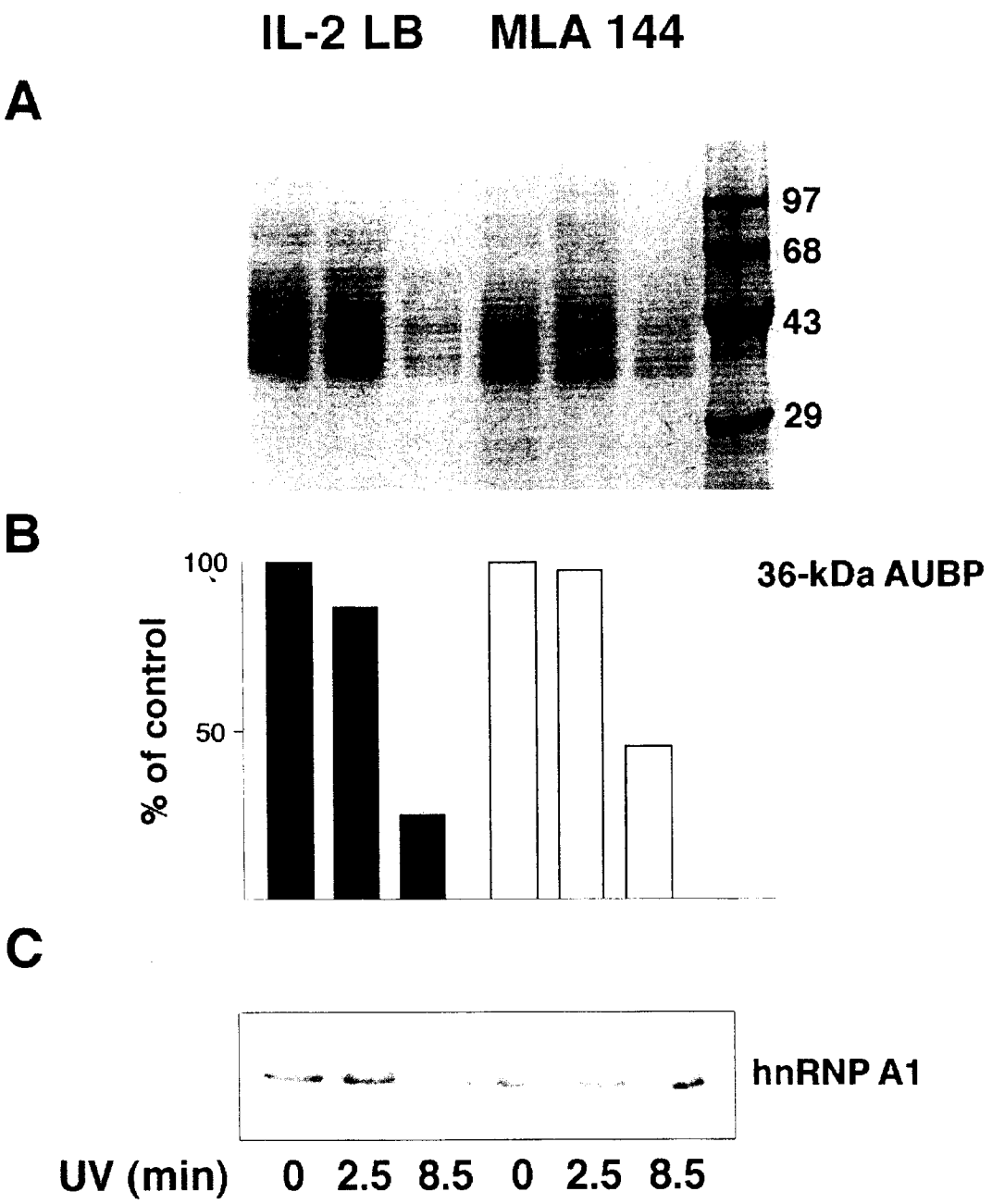

Figure 5. A) AUBP activities in the membrane-bound polysomal sub- fractions (MBPS) of UV-irradiated IL-2 lymphoblasts and MLA 144 cells. Each polysomal preparation (20 g of total protein) was analyzed in AUBP assay as described in the Methods section. Complexes were separated by 12.5% SDS-PAGE and visualized by autoradiography. Duration of *in vivo* UV exposure is indicated above each lanes. **B)** Densitometric analysis of the 36-kDa AUBP detected in the membrane-bound polysomal subfraction (MBPS) shown in Figure 5A. Values are shown as a percentage of the non-irradiated control. C) Detection of hnRNP A1 in the MBPS of IL-2 lymphoblasts and MLA 144 cells after various times of UV exposure *in vivo*.

examination of *in vivo* function of such cytoplasmic factors is the necessity for cell fractionation and extraction steps prior to *in vitro* analysis. Such procedures do not allow the preservation of structural architecture and therefore might be associated with the loss of critical information regarding key regulatory interactions. In order to overcome these difficulties, our aim was to design an experimental approach, using the combination of *in vivo* UV crosslinking and subsequent *in vitro* label transfer assay, in which the *in situ* RNA-AUBP associations could be retained and monitored. Irradiation of living cells with low intensity monochromatic (254 nm) UV light induces chemically reactive nucleotides which form covalent crosslinks with residues of virtually all amino acids of protein molecules that are in immediate contact with the nucleic acid at the time of UV exposure (Wagenmakers et al., 1980; Pashev et al., 1991; Pellé and Murphy, 1993; Pinol-Roma et al., 1989).

In this study we show that UV exposure of living cells results in a dose-dependent decrease of *in vitro* detectable AUBP activities, indicating effective generation of AUBP-RNA complexes by UV light within the cytoplasm. The ability of UV light exposure to induce AUBP-RNA complexes (as measured by *in vitro* analysis of AUBP activity) appeared to vary among cell types. Specifically, AUBP activity in MLA 144 cells was not as sensitive to UV crosslinking as compared with normal lymphoblasts. This finding suggests that a number of these proteins might be UV-crosslinked to ARE with lower efficiency *in vivo*. That it might indeed be the case is further supported by the Western blot results where the cytoplasmic lysate fractions of control and UV-exposed cells were assayed for known AUBP. Based on these results, we conclude that the UV light-induced decrease of *in vitro* AUBP activity did not arise from impaired extractability of AUBP from UV-exposed cells. Rather, these data indicate the presence of intrinsic RNA that covalently crosslinked to AUBP *in vivo*, masking its capacity to bind radiolabeled probe *in vitro*. The significance and practical applicability of our experimental approach is further strenghtened by recent studies of Scheper et al. They have demonstrated that an mRNA binding protein (Insulin-like growth factor II Cleavage Unit Binding Protein, ICU-BP) -with functional role in the cytoplasmic metabolism of IGF-II mRNAs- could be crosslinked to these RNAs *in vivo* and therefore depleted in the cytoplasmic lysates of those cells that had been transfected with sequences required for ICU-BP to recognize and cleave IGF-II mRNAs as monitored in a band-shift assay (Scheper et al., 1995; Scheper et al., submitted).

In addition, our results demonstrate that cytoplasmic AUBP are predominantly localized in the membrane-bound polysomal subfractions of both lymphoid cell types. This finding connects these proteins with a functional compartment, where critical regulatory steps of mRNA translation and/or degradation take place. Preferential distribution of various mRNA subsets among polysomal subfractions may contribute strongly to specific subcellular localization of their protein products (Hesketh and Pryme, 1991; Zambetti et al., 1990a and 1990b; Singer, 1992; Greene et al., 1976). Although there is no direct evidence resolving the subcellular localization of lymphokine mRNAs, we assume that, similar to other messages encoding for secretory proteins, lymphokine mRNAs may be preferentially recruited to the MBPS, allowing their translated proteins to be directly processed and targetted for excretion (Hesketh and Pryme, 1991). Direct examination of this question is very difficult, if not impossible, given the low abundance of lymphokine mRNAs and the extremely high RNase activity of lymphoid cells.

The observed differences in the extent of UV-crosslinkability of individual AUBP in normal lymphoblasts vs. transformed MLA cells as well as the non-uniform behavior of the various proteins during activation are difficult to interpret totally at this stage of investigation. In principle, the extent of UV light-induced complex formation is largely determined by the amino acid composition of the contact proteins or the nucleotide sequence of the interacting RNA ligand (Pinol-Roma et al., 1989). Therefore, evaluation of the detected changes in the UV-crosslinkability of individual AUBP cannot be directly addressed in the light of AUBP-ARE associations. On the other hand, fine alterations of AUBP-ARE interactions might well be part of a generally manifest abnormality in macromolecular associations in neoplastic cells (Ben-Ze'ev, 1985). Indeed, pathological stabilization of cytokine and

certain proto-oncogene mRNAs has been described in several tumor cell lines where alterations of regulatory *cis*-acting sequences of the messsenger RNA are not obvious (Schiavi et al., 1992; Ross et al., 1991). It might be interesting to note that in three additional tumor cell lines (Jurkat T cells, DP1727 erythroleukemia cell line and HEp-2 epithelial carcinoma cells) we found UV-crosslinkable pools of cytoplasmic AUBP similar in size to those of MLA cells, using other lymphokines as well as the *c-myc* proto-oncogene 3′ UTR-ARE as the RNA probe (not shown). Altogether, these results offer new aspects of the disordered mRNA metabolism in MLA cells where increased *in vitro* AUBP binding and affinity to a retrovirally altered ARE has been correlated to selective IL-2 mRNA stability *in vivo* (Henics et al., 1994).

In conclusion, these data indicate that association of cytoplasmic AUBP with RNA can be utilized to identify and examine these proteins as well as the functional significance of their molecular associations. Perturbations of AUBP-ARE interactions in tumor cells might contribute to pathologic mRNA metabolism. The advantages of this combined technique are that: i) it allows the preservation of intracellular nucleic acid-protein interactions apparent during complex regulatory processes and ii) it provides selective detection of the preserved alterations by means of specific RNA probes. Further data obtained through this approach might help us to estimate more accurately the *in vivo* interrelationships of RNA binding proteins with their RNA ligands during normal cellular processes and in tumorigenesis.

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INTERACTION OF AU-RICH SEQUENCE BINDING PROTEINS WITH ACTIN: POSSIBLE INVOLVEMENT OF THE ACTIN-CYTOSKELETON IN LYMPHOKINE mRNA TURNOVER

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Key words: actin-cytoskeleton, mRNA stability, lymphokine, lymphocyte activation, AU-rich sequences, AU-rich sequence binding proteins (AUBP).

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Running title: Actin-cytoskeleton and lymphokine mRNA turnover

SUMMARY

In this study we report that cytochalasin-induced disruption of microfilaments stabilizes lymphokine mRNAs in activated human peripheral lymphocytes. Parallel with this observation, a dose- and time-dependent increase in AUBP activities is apparent in the non-ionic detergent-resistant fractions of these cells, suggesting that cytochalasin-induced modulation of lymphokine mRNA stability might be mediated through cytoplasmic AUBPs. We provide evidence that some of the AUBPs can be immunoprecipitated with anti-actin antibodies, implicating that these proteins associate with the actin-based cytoskeleton *in vivo*. Moreover, disruption of the microfilament network by cytochalasins yields increased immunoprecipitable actin-AUBP complexes in the detergent-resistant cytoplasmic subfractions of lymphocytes. Additionally, we show that cytochalasin-induced changes in AUBP activities parallel with higher binding affinity of these proteins to ARE-containing RNA as judged by *in vitro* competition and *in vivo* UV-crosslinking analysis. Correlation of these findings with changes in mRNA stability indicates that the actin-cytoskeleton may play a physiologically important role in post-transcriptional regulation of lymphokine gene-expression during early lymphocyte activation.

Abbreviations: AUBP (AU-rich sequence binding protein), ARE (AU-rich sequence element), 3`-UTR (3`-untranslated region), PBMC (pheripheral blood mononuclear cell), HMG-CoA (3-hydroxy-3-methylglutaryl Coenzyme A), MBP (membrane-bound polysome), GAP (glyceraldehyde-3-phosphate), GAPDH (glyceraldehyde 3-phosphate dehydrogenase)

INTRODUCTION

Regulation of eukaryotic gene-expression is a complex process that requires well-synchronized coupling of events from nuclear gene-activation to cytoplasmic synthesis of gene products. Post-transcriptional modulation in the overall expression of a gene is a potent mechanism by which cells adjust the level of many transiently expressed factors, including cytokines (1-3), lymphokines (4-6), proto-oncogenes (7-9) or certain transcription-factors (10-14). These proteins are key regulators of proper cell growth and differentiation and are encoded by intrinsically labile mRNAs with specific AU-rich instability determinant sequences (ARE) in their 3` untranslated region (3`UTR) (15-16). Transient modulation of the turnover and/or translation of such mRNAs have been shown to be mediated in part by interactions of their *cis*-acting ARE sequences with specific AU-rich sequence element binding proteins (AUBPs) (17-21). Recently, many proteins capable of specifically binding to ARE have been identified in various cell types (22-27). Although, a number of attempts has been made to uncover molecular details about the nature and consequences of AUBP-ARE interactions, it is likely that no uniform mode of action can be applied to fully explain the mechanisms upon which these proteins function in the post-transcriptional metabolism of short-lived mRNAs (reviewed in 28).

Several lines of evidence suggest that cytoplasmic compartmentalization may contribute to appropriate post-transcriptional processing of various mRNAs (29-34). For example, specific distribution of α- and β-actin and *c-myc* mRNAs on polysomes within a well-defined subcellular compartment has been linked to their site-specific translation (33-36). Specific sequences

responsible for mRNA localization have been mapped within the 3`-UTR of these transcripts (33-36). The same portion of the mRNA has been implicated in specific polysomal and mitochondrial-cytoplasmic distribution of histone and the yeast tRNA processing enzyme, MOD5 mRNAs, respectively (37-38). Additionally, alternatively spliced HMG-CoA reductase mRNAs with various length 5`-UTR possess different polysomal distribution, suggesting the involvement of 5`-UTR sequences in selective localization in addition to its known influence on translation (39). These studies as well as other reports have also revealed that localized polysome assembly as well as sorting of various mRNAs require intact cytoskeletal cqmponents (40-42). Specifically, it is well documented that cytochalasin-induced disruption of the microfilament network releases mRNA from the polysomes and ceases translation (40, 43). Interestingly, removal of the 5`cap structure or the poly $(A)^+$ tract does not affect the association of mRNA with microfilaments, implying the role of coding-, 5`- or 3`-UTR portions of the message in this process, possibly through specific RNA-binding proteins (41).

Little is known, however, of how cytoarchitecture interact with unstable mRNA-AUBP complexes in regulated mRNA turnover. This might be pertinent during lymphocyte activation, when transient stabilization of various lymphokine mRNAs coincides with cytoskeletal rearrangements (44-46). Recently, we have shown that analysis of polysomal subfractions from various lymphoid cells revealed considerably more lymphokine-ARE specific AUBPs in the membrane-bound polysomal subfraction (MBPs) than in the non-membrane-bound fractions (47). This, together with the observation that MBPs strongly associate with the actin based cytoskeleton,

through a yet unclear mechanism (41), indicate that some of the AUBPs may play a role in post-transcriptional lymphokine mRNA metabolism in the context of microfilament-MBP complexes. In this study we test our hypothesis that the actin-cytoskeleton of lymphocytes should have links to ARE-AUBP mediated mRNA turnover. Our results indicate that the actin-based microfilament system may play a physiologically important role in post-transcriptional regulation of lymphokine gene-expression during early lymphocyte activation.

MATERIALS AND METHODS

Reagents. Actinomycin D (Act D), Cytochalasin B and D, rabbit antiserum raised against human actin, purified chicken gizzard actin, 2-mercaptoethanol and RPMI 1640 medium base were purchased from Sigma. Act D was kept at 4 ^{0}C as a 5 mg/ml stock solution. Phytohemagglutinin (PHA) was obtained from Wellcome Reagent Ltd., Beckenham, United Kingdom or from Sigma. [^{32}P]-UTP (3000 Ci/mmol) was either from Amersham or Izotop Kft. (Budapest, Hungary) and unlabeled nucleotides were obtained from Boehringer Mannheim. Guanidium thiocyanate was purchased from Fluka Biochemica.

Cell culture. PBMCs were separated from whole blood of healthy volunteers on discontinuous Ficoll-Paque gradient (Pharmacia Biothec) and cultured immediately in RPMI 1640 medium supplemented with 8 or 10% fetal calf serum (Protein GMK, Hungary). PHA was added to the cultures at a final concentration of 1 μg/ml.

Determination of mRNA stability. 30-50 million PBMCs were seeded at a density of 4 x 10^6 cells/ ml and activated in the presence of 1 μg/ml PHA for various periods of time. Cells were then exposed to Act D and DMSO (vehicle control) or cytochalasins (2.5 - 20 μM final concentration), harvested and their total cellular RNA was isolated by the single-step guanidium thiocyanate-phenol-chloroform extraction method (48) except that 0.1 volume of 2-mercaptoethanol was used in the denaturing solution. Equal amounts (5 - 10 μg/ lane) of RNA samples were size fractionated in 0.8% agarose-formaldehyde gel, blotted onto Hybond-N nylon membrane (Amersham Corp.) in 20 x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). Membranes were air dried and baked at 80 ^{0}C, 2 h *in vacuo*, then prehybridized for at least 12 h in 50% formamide, 0.8 M NaCl, 0.1 M PIPES, 0.1% Sarkosyl, 0.1 % Ficoll, 0.1 % polyvinylpyrrolidone, 0.1 % bovine serum albumin and 200 μg/ml sheared salmon sperm DNA at 42 ^{0}C. RNA was detected by hybridization of the membrane at 42 ^{0}C overnight in prehybridization mix containing 10 % dextran sulfate and 10^6 cpm/ml of [^{32}P]dCTP-labeled specific cDNA probes specific for IL-2, TNF-α or β2-microglobulin and generated by the random priming method (49). Blots were then washed three times at 56 ^{0}C in 0.1 x SSC containing 0.02 % sodium pyrophosphate and 0.5 % Sarkosyl. Blots were dried, exposed to Fuji RX film at -80 ^{0}C and autoradiograms analyzed densitometrically.

Preparation of cytoplasmic subfractions. Cytoplasmic subfractions were prepared as described (50) with minor modifications. 2-3 x 10^7 cells were washed in ice cold serum-free medium, pellets were resuspended in buffer A containing 10 mM PIPES, pH 6.8, 100 mM KCL, 2.5 mM $MgCl_2$, 300 mM sucrose, 1mM phenylmethylsufonyl fluoride (PMSF) and lysed on ice

for 3 min by addition of 1 % Triton X-100. Following centrifugation for 3 min at 900 x g, supernatants were collected, aliquoted and frozen immediately. Triton-insoluble pellets were extracted in buffer B containing 10 mM HEPES, pH 7.4, 15 mM NaCl, 1.5 mM $MgCl_2$, 1 % Tween-20, 0.5% sodium deoxycholate and 1 mM PMSF for 3 min on ice. Supernatants were collected after centrifugation with 1200 x g for 3 min at 4 ^{0}C.

RNA probes and in vitro label transfer assay. The 155-base RNA probe was generated as described (50) from a template encoding the proximal portion of IL- 2 mRNA 3`-UTR with four AUUUA motifs. The sequence of this probe is as follows: 5`-CCCU-n30-CU**AUUUAUUUA**AAU**AUUUAA** AUUUUAU**AUUUA**UUG-n75-CUUUUUGUAAG. 8 x 10^4 cpm [^{32}P]UTP-labeled probe RNAs (~10 fmol) were incubated with 2.5-10 μg proteins from cytoplasmic subfractions in 12 mM HEPES, pH 7.9, 15 mM KCl, 0.2 μM dithiothreitol, 0.2 μg/ml yeast tRNA and 10 % glycerol for 10 min at 30 ^{0}C. Protein-RNA complexes were fixed with UV light on ice using the UV Stratalinker model 1800 (Stratagene) (5 min, 3000 microwatts/cm^2) and exposed to RNase treatment (15 units of RNase T1 and 30 μg of RNase A/sample) for 15 min at 37 ^{0}C. Samples were then separated by 12.5% SDS-PAGE under reducing conditions, gels were dried and analyzed by autoradiography.

Immunoprecipitation. Protein A-Sepharose was preincubated with BSA, washed at low pH extensively, regenerated and conjugated with either polyclonal antibody against actin or with rabbit preimmune serum. Beads were then washed with buffer IP (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.5% Triton X-100, 1 mM PMSF and 1 μg/ml pepstatinA).

10 μg of detergent insoluble cytoplasmic subfractions were incubated with 3.2 x 10^5 cpm [^{32}P]UTP-labeled RNA probe, UV-crosslinked and digested with RNases as described above. Immunoprecipitation of RNA-protein complexes was performed in buffer IP at 4^0C by incubating the reaction mixture with the prepared beads for 45 min under gentle agitation. Beads were then pelleted and the supernatants were collected (depleted fractions). After washing extensively with buffer IP, beads were analyzed by 12.5 % SDS-PAGE and autoradiography.

In vitro competition experiments. Equal amounts of detergent-insoluble cytoplasmic lysate fractions were incubated with 8 x 10^4 cpm [^{32}P]-labeled RNA probe (~10 fmol) in the presence of various molar excess of unlabeled competitor RNA transcripts that had been generated by the addition of 2.5 mM each ATP, UTP, CTP and GTP in the transcription reaction. Labelled and cold RNAs were added simultaneously to the label transfer reaction, incubated with the lysates for 15 min, UV-crosslinked and RNase-treated as described. Complexes were analyzed by 12.5 % SDS-PAGE and autoradiography.

In vivo UV-crosslinking analysis. UV-irradiation of intact cells was combined with subsequent label transfer analysis essentially as described (47). Briefly, following activation and Act D/cytochalasin treatment, cells were pelleted, washed once in PBS and resuspended in ice cold PBS. Cells were distributed into 6-well tissue culture plates (1.6 x 10^7 cells/ well) and exposed to 254 nm UV light on ice under continuous gentle agitation using the UV Stratalinker 1800 (Stratagene) (3000 microwatts/cm^2) for 0 and 8 min. Cells were then harvested and cytoplasmic subfractions were prepared

as described above. Samples were then analyzed in *in vitro* label transfer assay. We have demonstrated earlier that this technique is capable to distinguish between AUBP pools with various degree of *in vivo* association with specific RNA sequences based on the assumption that *in vivo* covalently complexed AUBPs escape detection in a subsequent label transfer assay (47).

RESULTS

Cytochalaşin treatment stabilizes lymphokine mRNAs. To assess the effect of microfilament disruption on lymphokine mRNA stability, we activated freshly isolated PBMCs with PHA for 8 hours and exposed the cells to DMSO (vehicle control) or various concentrations of cytochalasins in the presence of Actinomycin D. We have tested Cytochalasin B and D and both agents were equally effective in our studies. Interleukin-2 and TNFα mRNA levels were monitored by Northern blot analysis. **Figure 1A**[1] demonstrates a representative of multiple experiments which were performed with at least four different cell batches. After 30 min of transcriptional blocade, IL-2 and TNFα mRNA levels were declined markedly, indicating unstable RNA species. When cells were exposed to Cytochalasin B at 2.5 or 5 micromolar concentrations, Act D treatment failed to decrease mRNA levels under a certain value, suggesting that IL-2 and TNFα mRNAs became more stable. However, the two mRNA species showed different stabilization response upon cytochalasin treatment. As

[1] Figures in this manuscript appear on pages which are not numbered, thus, Figure 1A, 1B, 2, 3, 4, 5A and 5B are on unnumbered pages 74-80, respectively in the Thesis.

half lives in response to cytochalasin as compared to Act D alone (**Figure 1B**). IFN-γ mRNA behaved similarly to that of IL-2 (not shown). β2 microglobulin mRNA was chosen as a control and no apparent change in its stability was detected in response to any treatment in the experiments. Interestingly, when PBMCs were activated for longer periods of time (> 20 h), IL-2 mRNA appeared to be already stable and we did not observe stabilization effect of cytochalasins (data not shown).

Effect of cytochalasin treatment on cytoplasmic AUBPs in activated PBMCs. To elucidate whether cytochalasin-induced lymphokine mRNA stabilization was accompanied by changes in the levels of cytoplasmic AUBPs in various subcellular fractions, we monitored the activity of these proteins in label transfer assays with a specific RNA probe encoding a portion of the 3`-UTR-ARE of IL-2 mRNA (see in Matherials and Methods). This RNA probe detected 4 major AUBPs in the Triton X-100-soluble fraction (90-, 70-, 50-, 41-kDa) and 4 in the detergent-insoluble compartment (70-, 50-, 41- and 36-kDa) of PBMCs activated for 8h (**Figure 2**). Marked dose and time dependent increase in AUBP activities was observed following Cytochalasin D treatment in the detergent-resistant fractions, but not in the detergent soluble fractions (**Figure 2A** and **B**). Among the detected AUBPs, the 36- and 41-kDa proteins appeared to be affected the most, and the appearance of a 60-kDa complex was observed after 2-3 h treatment (**Figure 2B**). We were interested to see if there were comparable changes in the activity of these proteins in similar subcellular fractions of PBMCs activated for longer periods of time. At >20 h of activation, cytochalasin treatment failed to cause any detectable changes in AUBP activities in either of the two subfractions (data not shown).

Immunoprecipitation of AUBP complexes with anti-actin antibodies. From the experiments described above, we concluded that cytoplasmic AUBPs might be in spatial and functional association with cytochalasin sensitive structures during specific phases of lymphocyte activation. To test this assumption, we attempted to immunoprecipitate RNA-AUBP complexes using specific antibodies to actin. **Figure 3** shows that a dominant 36-kDa AUBP complexed with anti-actin antibodies from the Triton X-100-insoluble cytoplasmic subfraction of activated PBMCs. The difference in AUBP activity in the control vs. cytochalasin-treated subcellular fractions was retained in the immunoprecipitated AUBP fractions (**Figure 3B, lanes 5-6**). At this point, it was important to ascertain that actin itself did not bind AU-rich RNA. Purified chicken gizzard actin was tested in a label transfer assay using various ARE-containing RNA probes and no complex formation was detected in any case (not shown). However, in fractions, immunoprecipitated prior to the label transfer assay, we also detected efficient binding of a 36-kDa complex to AU-rich RNA (not shown). These data implicate that some RNA-AUBP complexes are in strong association with actin in the extracted subcellular fractions through the protein partner of the complex as well as demonstrate that this association is permissive for these AUBPs to bind ARE sequences *in vitro* (see Discussion).

Effect of cytochalasins on in vitro AUBP binding affinity and in vivo UV-crosslinkability to ARE. In order to gain more details of how cytochalasin-mediated effects influence AUBP-RNA interactions, first we monitored *in vitro* AUBP binding affinity to ARE-containing RNA probes in a competition assay. **Figure 4** demonstrates that cytoplasmic AUBPs

from the detergent-resistant fractions of cytochalasin-treated lymphocytes bind IL-2 3`-UTR ARE with considerably higher affinity as compared to those of control cells. This result suggests that microfilament disruption *in vivo* facilitates *in vitro* association of AUBPs with specific ARE sequences within the extracted cytoplasmic fractions. Individual AUBPs, however, behaved disssimilarly in this context, indicating their different susceptibility to the treatment and/or association with the RNA *in vivo*. To test if these changes in *in vitro* AUBP binding affinities were reflected in the *in vivo* association of any of these proteins with ARE sequences, we exploited the capability of a technique, described recently in our laboratory, to detect such parameters (47). In agreement with our *in vitro* results, a dominant 36- and a less apparent 50-kDa AUBP complex could be UV-crosslinked to RNA in cytochalasin-treated lymphocytes with higher efficiency *in vivo*, suggesting their closer association with ARE following microfilament disruption (**Figure 5**).

DISCUSSION

In this study we made attempts to better characterize basic mechanisms of how lymphokine mRNA stability is modulated and to find potential links between ARE-AUBP mediated mRNA turnover and changes in cytoarchitecture upon lymphocyte activation. Two distinct lines of evidence prompted us to test possible microfilament involvement in mRNA-AUBP interactions and consequently, in lymphokine mRNA stability. First, coordination of cellular events brought upon by lymphocyte activation has been proposed to occur in part with rearrangement of cytoskeletal structures

(51-54). Development of normal "capping" process, for example, can be prevented by microfilament disrupting agents such as cytochalasins (44-46). Moreover, key mediators in mitogenic stimulation-triggered signal transduction, such as protein kinase C, have been documented to associate with cytoskeletal components (53, 55-57). Thus, lymphocyte cytoarchitecture might be an essential element in normal progression through activation. Second, subcellular localization of many mRNA species has been implicated in proper spatial expression of the encoded gene product (29-32). *c-myc* protein, for example, appears to be synthesized more efficiently on polysomes located on the perinuclear cytoskeleton as it has to be transported back to the nucleus following translation (33-34). Similarly, lymphokine mRNAs are thought to be translated primarily on membrane-bound polysomes, from where the proteins can easily be recruited to the endoplasmic reticular and Golgi compartments for continuous processing and excretion (47). Polysomal anchorage to cytoskeletal structures seems to be generally important in efficient translation of mRNAs (41, 43). Since stability of many mRNA species is closely linked to their translation, it is conceivable to assume that specific association of the mRNA with cytoskeletal elements may, in turn, contribute to the regulation of its turnover. These observations raise a number of pertinent questions. Do lymphocyte cytoskeletal elements contribute to selective targeting, and possibly to specific turnover regulation of various mRNA species? Do these cells take advantage of structure-based regulatory functions evident in cell types that possess considerably more extensive cytoskeletal substance?

Our attempts to examine the effect of microfilament disruption on lymphokine mRNA turnover revealed that IL-2, TNFα and IFN-γ (not shown) mRNAs were stabilized when activated lymphocytes were exposed to cytochalasins (Figure 1). This effect was observable at early periods of activation (6-8 h) and not at later (> 20 h) time points. This would indicate that cytochalasin-sensitive structures are involved in regulated mRNA stability during early T cell activation, when lymphokine mRNAs are still unstable (58). After 20 h of mitogenic activation, when lymphokine mRNAs become transiently stabilized, we could not detect cytochalasin-directed effects at the mRNA or AUBP activity level. Therefore, it is possible that microfilament-based structures are parts of a mechanism which underlies the transition from transcriptional to post-transcriptional means of lymphokine production, leading eventually to down-regulation of lymphokine gene-transcription and manyfold stabilization of lymphokine mRNAs (58). Cytochalasins disorganize the actin bundles by reversibly binding to the barbed end of the filament, and possibly also sever preformed F-actin based structures. Disruption of the lymphocyte microfilament scaffold may result in unregulated early lymphokine mRNA stabilization and consequently, in abnormal lymphokine production by these cells. The relevance of this speculation may especially be emphasized in the case of hematopoietic tumor cells where aberrant stabilization of various cytokine mRNAs were observed without apparent sequence alterations in their 3`-UTR instability determinant elements (59). On the other hand, structural alterations in the organization of cytoskeletal matrix seem to be a general phenomenon apparent in transformed cells (60-61). Our results, showing that IL-2 mRNA became more stable upon cytochalasin-treatment -as compared to that of TNFα-, suggest different association of this two mRNA

species with cytochalasin-sensitive structures and/or that their stability might involve different mechanisms at this stage of activation. The primary scope of this study was to test if lymphokine mRNA stability, thought to be mediated by ARE-AUBP interactions, involved any elements which were related to the actin-cytoskeleton. We found that cytochalasin-induced stabilization of various lymphokine mRNAs correlated with increased AUBP activities in the non-ionic detergent-resistant cytoplasmic subfractions and not in the detergent-soluble fractions of cells exposed to the microfilament disrupting agents (Figure 2). Importantly, we detected these changes at<8 h of activation and not at later time points. Therefore, increased AUBP activities in these cytoplasmic fractions coincided with increased mRNA turnover.

Our immunoprecipitation experiments revealed a predominant 36-kDa complex in close association with actin or actin-containing structures in the detergent-resistant cytoplasmic fraction of activated lymphocytes. This finding indicates that AUBP activities co-localize with F-actin in lymphocytes (Figure 3). Moreover, this finding is novel in that it describes a molecular assembly in which a lymphokine 3`-UTR-ARE associates specifically with actin-containing structures through its *trans*-acting AUBP regulator(s). Two proteins with near identical molecular mass has been identified within this complex in activated T lymphocytes as being heteronuclear ribonucleo-protein A1 (hnRNP A1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (24, 62). Additionally, GAPDH, along with additional members of the glycolytic cascade, is spatially localized to actin-based microfilaments in virtually all eukaryotic cells (63-65). Taken these observations together, it is tempting to speculate that certain locations

of the actin-cytoskeleton may serve as regulatory switching points where multifunctional RNA-binding enzymes, such as GAPDH receive multiple metabolic signals to prompt the enzyme to perform one metabolic function over the other. Intriguingly, Bassell et al. demonstrated that visualization of single mRNAs by ultrastructural *in situ* hybridization reveals most of the poly $(A)^+$ mRNA at actin filament intersections in fibroblasts (66). As of importance, it has been shown that the substrate, glyceraldehyde-3-phosphate (GAP) dissociates GAPDH from actin (67). The *in vitro* competition results, in accordance with the *in vivo* UV-crosslinking data presented here, demonstrate that disruption of the actin-based component of the actin-AUBP-ARE assembly results in altered capability of some of these AUBPs to associate with the RNA (Figure 4 and 5) and may, therefore, influences the association of the mRNA itself with the structural scaffold. Nevertheless, these data strongly implicate cytoplasmic microfilaments in the regulation of ARE-AUBP interactions and consequently, in AUBP-mediated lymphokine mRNA turnover.

In conclusion, we demonstrated that disruption of the actin-based microfilament network in lymphocytes during early activation leads to stabilization of lymphokine mRNA species. This phenomenon is paralleled with increased AUBP activity in the Triton X-100-insoluble cytoplasmic subfraction of these cells. The association of these AUBPs with ARE is augmented as revealed in *in vitro* competition studies and by the combined *in vivo* UV-rosslinking - *in vitro* label transfer method. Increased AUBP activity within the dominant 36-kDa complex was apparent in immunoprecipitation experiments, indicating that cytochalasin-derived effects on lymphokine mRNA stability might, in part, be mediated by

alterations of molecular associations between AUBPs and actin-based structures. Although, these results strongly suggest structural and functional connection between cytoplasmic microfilaments and lymphokine mRNA stability, further studies will be needed to uncover key aspects of the involved mechanisms.

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FIGURE LEGENDS

Figure 1. ***Effect of cytochalasin treatment on lymphokine mRNA stability.*** **A)** Northern blot analysis of total cellular RNA extracted from human PBMCs that had been activated with PHA for 8 h and exposed to the transcriptional inhibitor, Actinomycin D (5 μg/ml) and to the microfilament disorganizing agent, Cytochalasin B ("+" = 2.5 μM, "++" = 5.0 μM). 10 μg of total RNA was loaded in each lane. **B)** Densitometric analysis of the Northern blot shown in Figure 1A. Values were normalized for the loading control and expressed as percentage of the untreated control. **1:** untreated control, **2:** Act D (5 μg/ml), **3:** Act D and Cytochalasin B (2.5 μM), **4:** Act D and Cytochalasin B (5 μM).

Figure 2. ***Analysis of AUBP activities in cytoplasmic subfractions of cytochalasin-treated lymphocytes.*** 2.5 μg total proteins of Triton X-100-soluble (**A**) and insoluble (resistant) (**B**) cytoplasmic fractions of PHA-activated (8 h) human PBMCs were incubated with ^{32}P-UTP-labeled AU-rich RNA probe (see in Materials and Methods) and UV-crosslinked. Following RNase treatment, samples were analyzed by 12.5% SDS-PAGE and autoradiography. **1:** untreated control, **2:** DMSO (vehicle control), 3h, **3:** 5 μM Cytochalasin D, 1h, **4:** 10 μM Cytochalasin D, 1h, **5:** 20 μM Cytochalasin D, 1h, **6:** 10 μM Cytochalasin D, 2h, **7:** 10 μM Cytochalasin D, 3h.

Figure 3. ***Immunoprecipitation of cytoplasmic fractions with anti-actin antibodies.*** **A)** Coomassie Blue-stained loading control of total Triton X-100-insoluble cytoplasmic fractions of activated human PBMCs. **1:** DMSO

control, **2:** 10 μM Cytochalasin B-treatment for 2 h. One-fourth of the immunodepleted fractions after immunoprecipitation with Protein A-Sepharose immobilized anti-actin immuneserum (**3** and **4**), and immunoprecipitated fractions with anti-actin immuneserum or rabbit preimmuneserum (**5-6** and **7-8**, respectively) were analyzed by 12.5% SDS-PAGE. **B)** Autoradiographic detection of immunoprecipitated ARE-AUBP complexes. Lanes are identical to those of Figuer 3A.

Figure 4. ***Analysis of the effect of cytochalasin treatment on AUBP binding affinity to AU-rich RNA in vitro.*** Triton X-100-insoluble cytoplasmic frąctions (2.5 μg total protein) of activated and DMSO- (**left panel**) or Cytochalasin B-treated (10 μM, 2 h) (**right panel**) human PMBCs were analyzed in a competition label transfer experiment. Increasing molar excess of the unlabeled (cold) competitor is indicated on the top of each panel.

Figure 5. ***Effect of cytochalasins on the in vivo UV-crosslinkability of cytoplasmic AUBPs to ARE-containing RNA.*** **A)** 8 h PHA-activated, and DMSO (**left panel**) or Cytochalasin B-treated (10 μM, 2 h) (**right panel**) human PBMCs were exposed to monochromatic UV light (see in Materials and Methods) for 0 or 8 min. Triton X-100-insoluble cytoplasmic fractions were analyzed in a label transfer assay as described. **B)** Intensities of the dominant 36-kDa and a less apparent 50-kDa complex were quantitated by densitometry and relative values were expressed as the percentage of non-UV-treated samples. Duration of *in vivo* UV exposure is indicated below each panels.

A.

Act D	-	+	+	+
Cyt-B	-	-	+	++

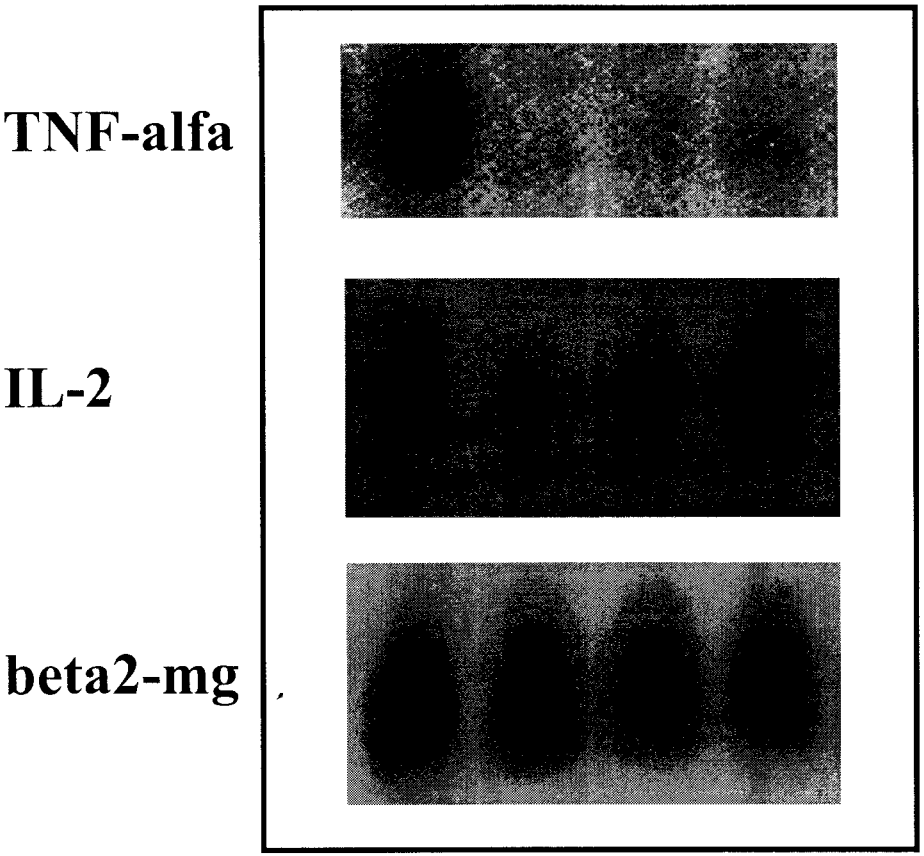
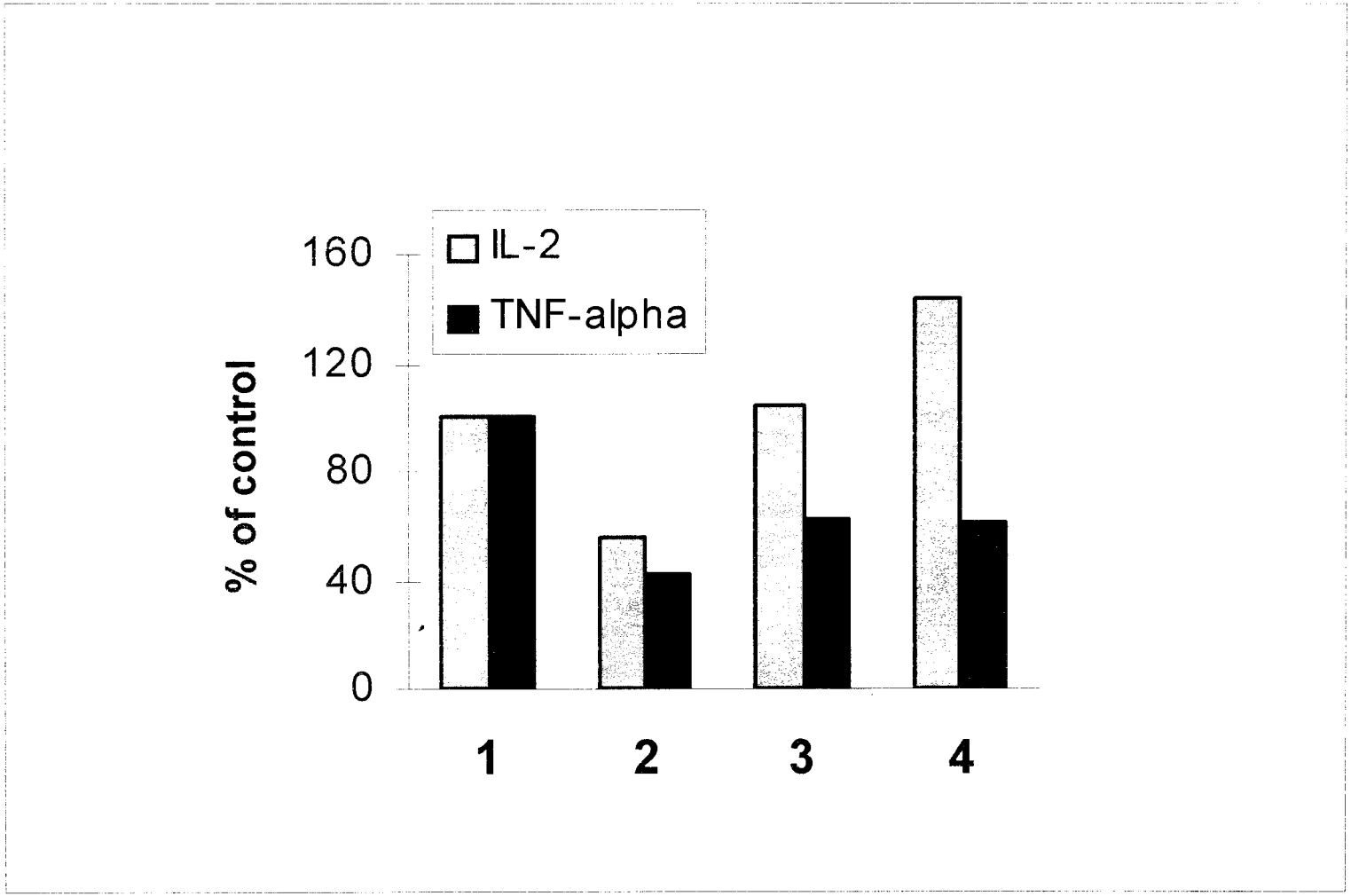
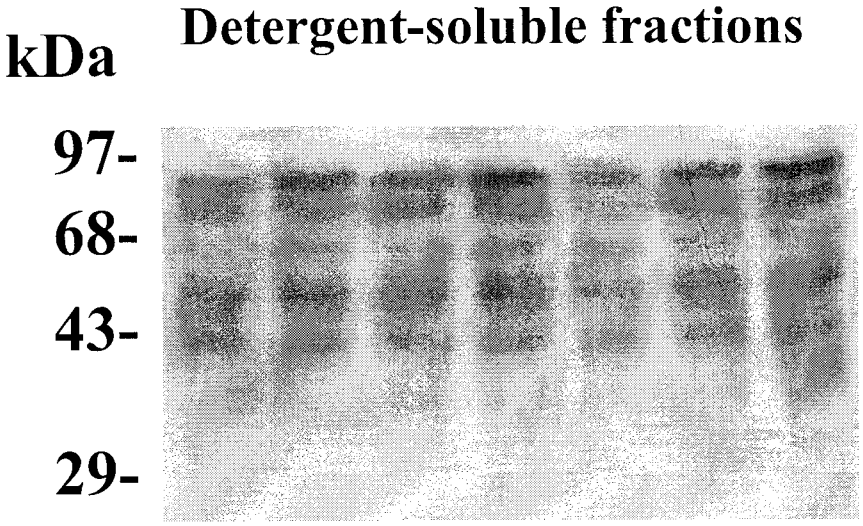


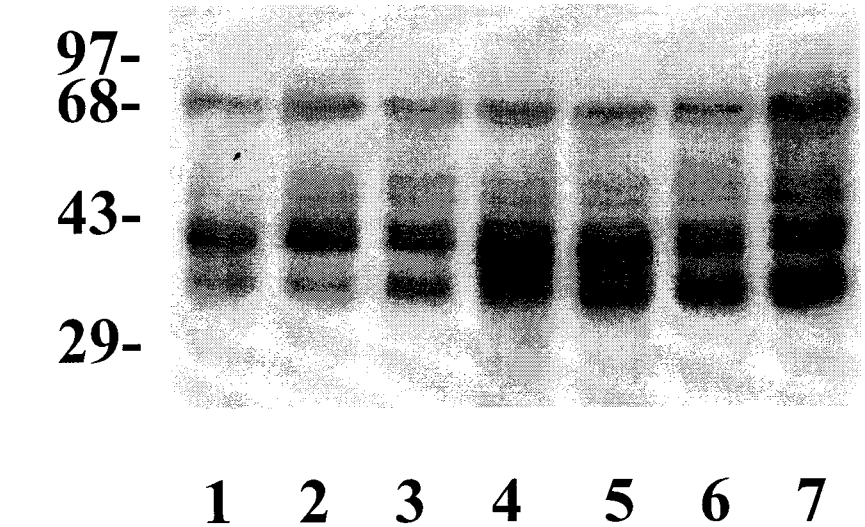
Fig. 1 B

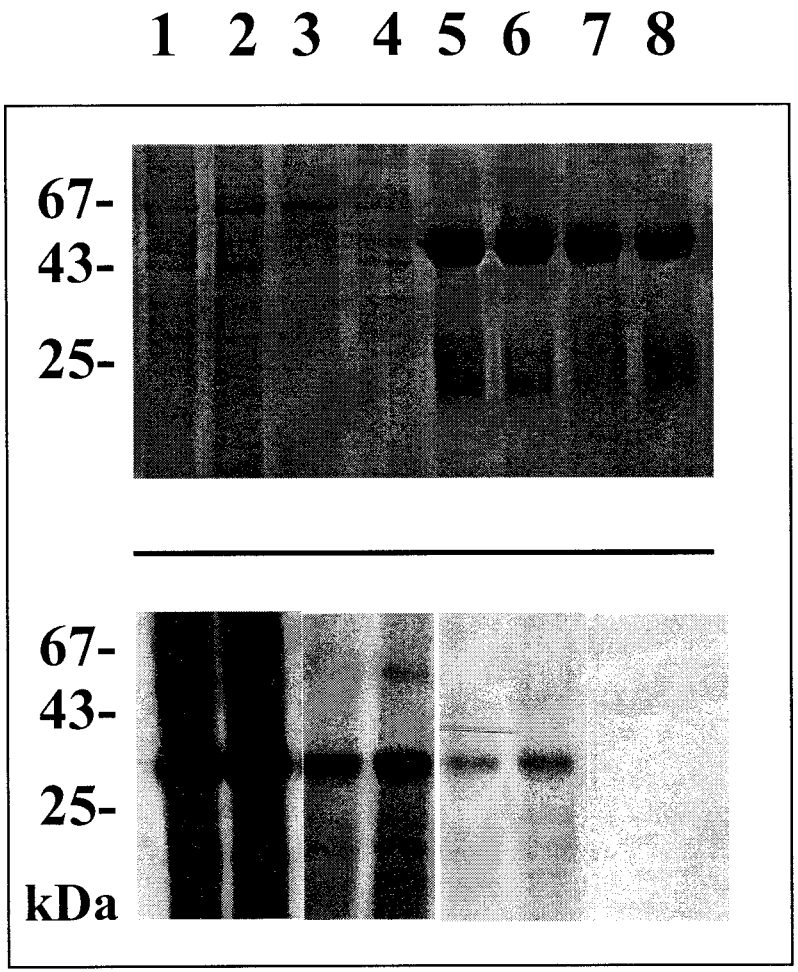


A.

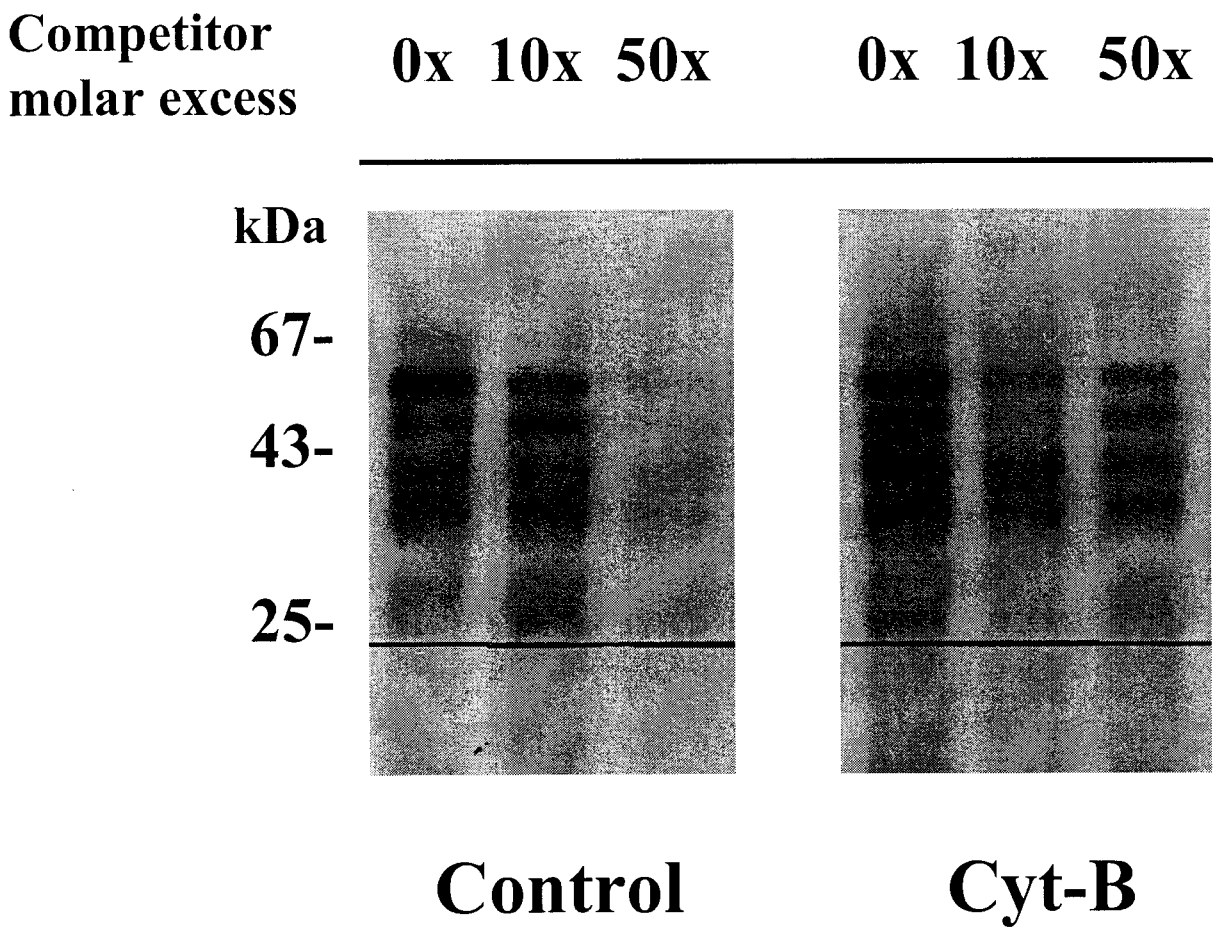

kDa
Detergent-soluble fractions
97-
68-
43-
29-

B.


Detergent-resistant fractions
97-
68-
43-
29-
1 2 3 4 5 6 7


1 2 3 4 5 6 7 8
A.
67-
43-
25-
B.
67-
43-
25-
kDa

A.


Competitor molar excess
0x 10x 50x
0x 10x 50x
kDa
67-
43-
25-
Control
Cyt-B

A.

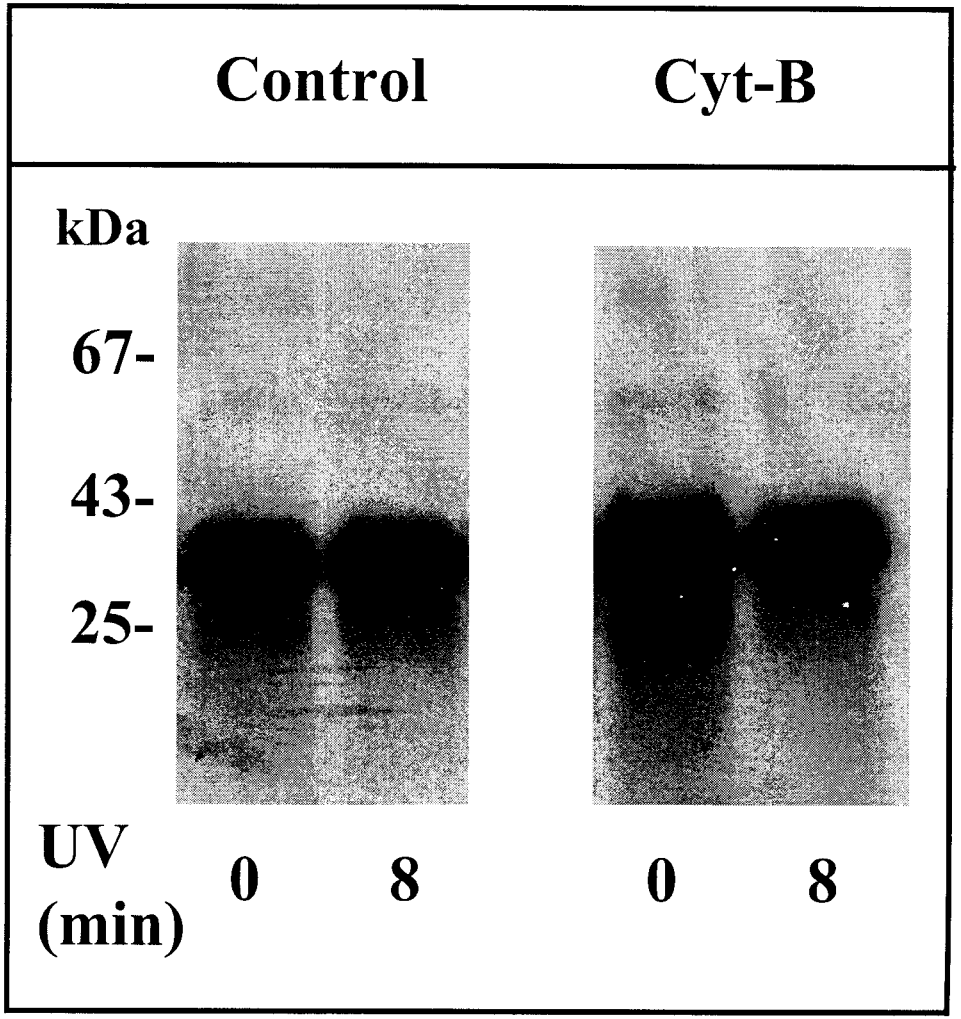
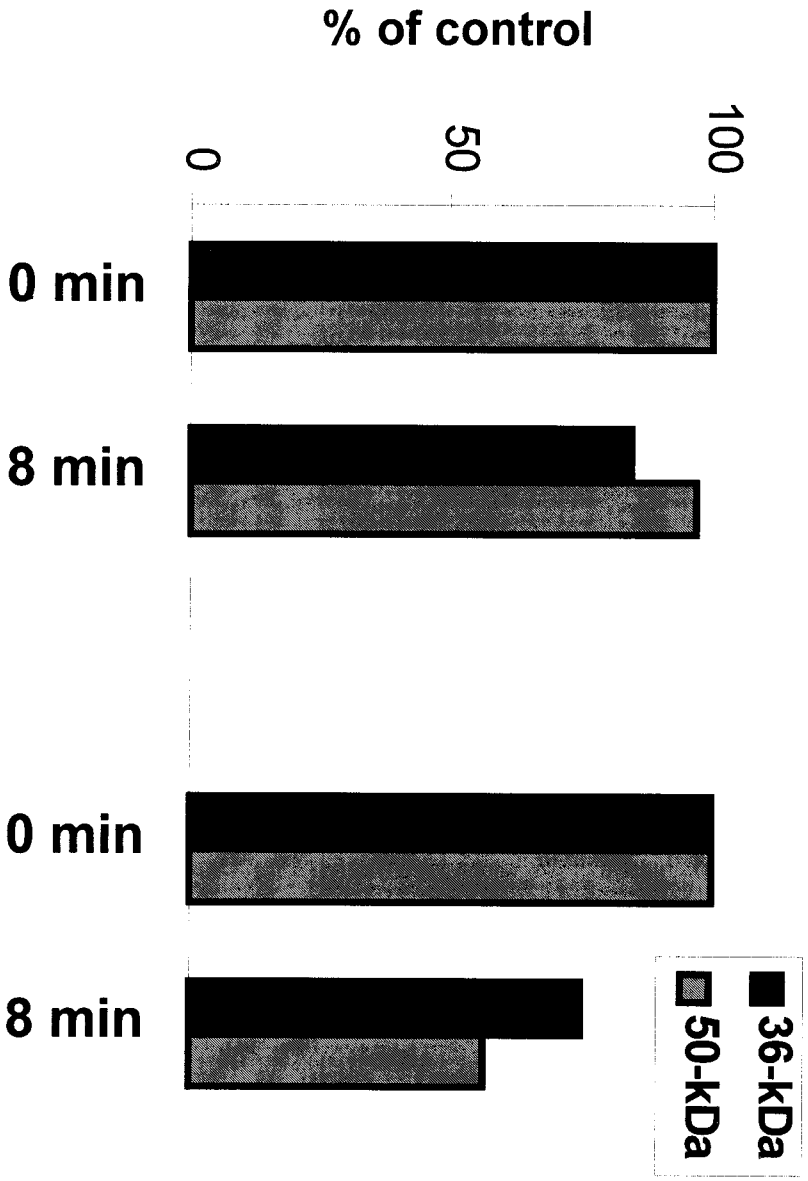


Fig. 5 B.



Chapter 5: SUMMARY AND CONCLUDING REMARKS

The molecular aspects of lymphokine mRNA metabolism offers many general mechanisms with relevance to post-transcriptional regulation of eukaryotic gene-expression. Large body of evidence has accumulated during the past five years indicating that critical regulation at the post-transcriptional level contributes to the appropriate cellular responses to a variety of metabolic signals (56,57,58,59,60). Failure of this kind of cellular adaptation may often result in the onset of malignant transformation and maintenance of the malignant phenotype (40,41). Therefore, it is extremely challenging to uncover yet unresolved molecular details of these critical cellular processes. The majority of studies involves *in vitro* characterization of RNA-protein interactions that occur between proteins of cell extracts and synthetic RNA probes. Although, the value of such systems cannot be emphasized enough, the interpretation of the results obtained is hampered by the "test tube" nature of information. It is difficult to judge to what degree the molecular associations and regulatory mechanisms -which may otherwise be relevant *in vivo*- are lost during biochemical dissection of the components. Thus, experimental models seeking to overcome such difficulties may provide the possibility of opening new routes in the investigation of mRNA-protein interactions apparent in complex regulatory processes. By using the presented model systems and the outlined experimental regimes, I arrived to the following conclusions:

1. Studying the MLA 144 system was a valuable tool to gain insight into the molecular details of how lymphokine production is modulated post-transcriptionally. Additionally, it allowed me to uncover several basic aspects of how the stability of IL-2 mRNA is

regulated in particular. *For the first time, I was able to draw correlation between binding affinity of cytoplasmic AUBPs to a retrovirally modified lymphokine mRNA (IL-2) ARE in vitro and stability of the same lymphokine mRNA in vivo. I could identify some of these proteins as members of the most abundant nuclear protein family, hnRNP A1 and C as well as determine their sub-cytoplasmic distribution in various lymphoid cells. This system allowed me to perform analysis of how the macromolecular components interact in vitro.*

2. We were able to develop a novel experimental technique to analyze lymphokine mRNA-ARE - AUBP interactions *in vivo* in various lymphoid cells. I could monitor specific changes of such associations during lymphocyte activation. *By comparing normal and transformed cells, I concluded that substantial differences in RNA-protein interactions were apparent, possibly as a result of a generalized problem at the level of macromolecular interactions in malignant cells. By the aid of this new technique, I was able to show an enrichment of cytoplasmic AUBPs in a functional compartment, the membrane-bound polysomal subfraction of lymphoid cells.*

3. I presented evidence that mRNA stability is closely linked to the cytoskeleton of lymphocytes. *Disarrangement of the actin-based microfilament network results in selective lymphokine mRNA stabilization in close correlation with specific changes of cytoplasmic AUBPs in the detergent-resistant subfraction. I showed that some of the ARE-AUBP complexes are in close association with actin through the protein partner of the complex. Using multiple approaches, I*

demonstrated that changes in the affinity of AUBPs to ARE both in vitro and in vivo parallel with changes in lymphokine mRNA stability induced by microfilament disruption.

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