Association of Heterogeneous Nuclear Ribonucleoprotein A1 and C Proteins with Reiterated AUUUA Sequences*

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Post-transcriptional regulatory mechanisms have been shown to play a major role in gene expression in eukaryotic cells. The presence of a reiterated pentamer (AUUUA) in the 3′-untranslated region (UTR) of mRNAs encoding lymphokines, cytokines, transcription factors, and proto-oncogenes has been shown to be associated with rapid turnover and translation attenuation. Cytoplasmic proteins (70, 50, 43, 36, and 25 kDa) capable of specifically binding to RNAs containing these AU-rich sequences were identified in human peripheral blood T lymphocytes. Levels of the 36-kDa protein were markedly increased following transcriptional, but not translational inhibition, a feature recently reported for hnRNP A1, a protein of comparable mass. Antibodies directed against heterogeneous nuclear ribonucleoproteins (hnRNPs) A1 and C immunoprecipitated 36- and 43-kDa proteins that had bound the AUUUA-rich region contained in the 3′-UTR of granulocyte-macrophage colony-stimulating factor mRNA. Recombinant hnRNP A1 was shown to preferentially bind to RNAs containing AUUUUA sequences in a specific manner, and displayed comparable patterns to the 36-kDa AU-specific binding proteins following partial proteolysis. These data identify for the first time hnRNP A1 and C as cytoplasmic proteins in human lymphocytes that are capable of specifically associating with reiterated AUUUUA sequences present in the 3′-UTR of labile mRNAs. As such, they may play a role as trans-acting factors in the modulation of cytoplasmic mRNA turnover and translation, in addition to their previously characterized roles as pre-mRNA binding proteins involved in nuclear mRNA processing.

In eukaryotic cells, the regulation of mRNA turnover is presumably permits eukaryotic cells nontranscriptional means to modulate the expression of genetic information. Various environmental stimuli (heat shock, viral infection, growth stimulation) can result in substantial changes in the turnover and translation rates of specific mRNAs (1). The role of trans-acting proteins in mediating post-transcriptional gene expression through binding to common cis-acting mRNA sequences has been compellingly demonstrated in the regulation of cellular iron metabolism. In this system, coordinate reciprocal changes in translation and mRNA stability are mediated by a single protein (iron-response binding protein) capable of binding to a specific iron-response element located in the 5′ and 3′ ends of the ferritin and transferrin receptor mRNAs, respectively (2–4).

A variety of proto-oncogene, transcription factor, and lymphokine genes encode mRNAs distinguished in their cytoplasmic lability (half-life < 30 min). Many of these unstable mRNAs contain reiterations of a specific sequence (AUUUA) in their 3′-untranslated region (3′-UTR) (5, 6). Reiterations of this sequence are capable of conferring instability on a previously stable mRNA in heterologous gene constructs (6). Other studies have implicated these sequences in regulating translation of mRNA (7, 8). The characterization of AU-rich motifs as cis-acting sequences which regulate mRNA stability has prompted investigation of the trans-acting factors that recognize and bind these sequences. Using differing cell lines and preparations (nuclear versus cytoplasmic) a variety of AU-specific binding proteins (AUBPs) have been described (9–13). Malter and co-workers (9) have described a cytoplasmic AUUUA-specific mRNA-binding protein of 98 kDa (denoted AUBF) in T lymphocyte cell lines and peripheral blood mononuclear cells. Using human T lymphocytes, Bohjanen (11, 12) has described three AUBPs in human T lymphocytes, AU-A, a cytoplasmic/predominantly nuclear 34-kDa protein, as well as AU-B (30 kDa) and AU-C (43 kDa) which are cytoplasmic in location and induced with activation. Vakalopoulou (13) described a 32-kDa AUBP present in nuclear and cytoplasmic extracts of HeLa cells and demonstrated a correlation between binding of this protein and reduced accumulation of both nuclear and cytoplasmic mRNA. This group also identified an 38-kDa nuclear AUBP as hnRNP C, but concluded that the 32-kDa protein was not an hnRNP A protein (13).

We now present evidence that human peripheral blood lymphocytes contain several cytoplasmic proteins (70, 50, 43, 36, and 25 kDa) capable of specifically binding to RNAs containing these AU-rich sequences. Levels of the 36-kDa protein were markedly increased following transcriptional, turnover...
but not translational inhibition, a feature recently reported for hnRNP A1, a protein of comparable mass. Immunoprecipitation with specific antibodies demonstrated that the 36- and 43-kDa proteins share antigenic reactivity with hnRNP A1 and C. Recombinant hnRNP A1 demonstrated similar AU binding activity as the putative 36-kDa protein. To our knowledge, these data represent the first identification that some cytoplasmic AUBPs are members of the family of hnRNPs, and combined with the previous studies suggest the role of hnRNP A1 in the regulation of mRNA turnover and translation. Finally, these data implicate hnRNPs in cytoplasmic regulation of RNA metabolism in addition to their well-recognized roles in pre-mRNA processing and splicing.

**EXPERIMENTAL PROCEDURES**

**Materials**—Actinomycin D, cycloheximide, 5,6-dichloro-1-ribofuranosylbenzimidazole (DRB), and trypsin were purchased from Sigma and freshly made up prior to use, except for actinomycin D, which was prepared and stored as a stock solution (5 mg/ml, 4°C). Monoclonal antibodies SP20, 4F4 (anti-hnRNP C), and 9H10 (anti-hnRNP A1) were generously provided by Dr. Gideon Dreyfuss as recombinant human hnRNP A1 and C (14-18). [α-32P]UTP (3000 Ci/mmol) was purchased from Amersham, while unlabeled nucleotides were obtained from Boehringer Mannheim.

**Cell Culture and Lysate Preparation**—Human peripheral blood mononuclear cells obtained from volunteer donors by leukapheresis were isolated by Ficoll-Hypaque discontinuous gradient centrifugation and cultured at 2 × 10⁶ cells/ml in RPMI 1640 medium (KC Biologicals, St. Louis, MO) supplemented with 5% heat-inactivated (56°C, 1 h) fetal bovine serum (Sigma) and 50 μg/ml gentamicin sulfate (U. S. Biochemical Corp.) at 37°C in a humidified atmosphere of 5% CO₂ in air. Cells were stimulated with that concentration of PHA (1 μg/ml, Wellcome Reagent Ltd., Beckenham, United Kingdom) found to cause maximal stimulation.

Cytoplasmic lysates were prepared by washing the cells twice in ice-cold phosphate-buffered saline, followed by gentle resuspension of the cell pellet in 1% Triton X-100 lysis buffer containing 10 mM PIPES, pH 6.8, 100 mM KCl, 2.5 mM MgCl₂, 300 mM sucrose, 1 mM phenylmethylsulfonyl fluoride and incubation for 3 min on ice followed by a 3-min centrifugation at 500 × g (19). Incubation of the cell pellet in lysis buffer from 1 to 10 min prior to centrifugation did not alter the profile of AUBP detected by this method, nor did the inclusion of leupeptin (1 μg/ml) in the lysis buffer. Cell clumping was not observed. The use of this lysis buffer and method was chosen as it was shown to leave the cytoskeleton intact with minimal polyinosine disruption (19). This extraction technique was slightly modified as the lysis buffer lacked 1 mM CaCl₂ and included simultaneous exposure of the cell pellet to the detergent and the other components of the lysis buffer. In multiple experiments, each of these modifications was shown not to affect the levels of the 25-, 36-, and 43-kDa AUBPs in cytoplasmic lysates relative to the original method (19), nor were levels of AUBP altered in comparison to lysates prepared with Nonidet P-40-containing lysis buffers (9). These data are consistent with observations in the original report that the presence of calcium in the lysis buffer was shown not to be essential (19).

**RNA Probes and AUBP Assay**—The Δ2R1 probe, which contains a sequence found in the 3'-UTR of GM-CSF c-mRNA (9), was prepared by T7 RNA polymerase transcription of EcoRI-linearized pT7/Tsc19 plasmid with 4 AUUUA sequences in the BamHI site of GM-CSF mRNA (9). After coincubation of the Δ2R1 probe and cytoplasmic lysates from resting and activated cells with [32P]UTP (3000 Ci/mmol) from Amersham, 0.0125 mM UTP, 2.5 mM ATP, GTP, and CTP from Boehringer Mannheim. The AUUUA sequences in each RNA are underlined in Fig. 1. RNA probes (8 × 10⁶ cpm) were incubated with 10 μg of RNase A for 30 min at 37°C. The sample was then analyzed under denaturing conditions by 15% SDS-PAGE, followed by autoradiography.

Immunoprecipitation of AUUUA-binding Proteins with Monoclonal Antibodies to HnRNP C and A1 Proteins—Human lymphocyte cytoplasmic lysates were incubated with [α-32P]Δ2R1 RNA, UV cross-linked, and digested with RNase as described above. The RNA–protein complexes were added to 1.5 ml of SP20 (negative control antibody), 4F4 (anti-hnRNP C), or 9H10 (anti-hnRNP A1) ascites in 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.5 mM MgCl₂, 0.5% Triton X-100, 2 μg/ml each aprotinin, leupeptin, and pepstatin A, and 1 mM PMSF and incubated 2 h at 4°C. 5 μg of goat antimouse F(ab')₂, and 25 μl of protein A-Sepharose beads (Pharmacia LKB Biotechnology Inc.) were added and incubated with gentle mixing for 1 h at 4°C. Beads were pelleted by brief centrifugation, washed 5 times in phosphate-buffered saline, boiled in 10 μg of protein from cytoplasmic lysates of PHA-stimulated cells, and analyzed by SDS-PAGE. Using this method, 9H10 antibody was capable of completely immunoprecipitating 1 μg of recombinant hnRNP A1, with no evidence of cross-reactivity with SP20 and 4F4 antibodies.

Analysis of AUUUA-binding Proteins by Particulate-Proteolysis—Cytoplasmic lysates (5 μg) prepared from 20-μg PHA (1 μg/ml) culture (19) were treated with recombinant hnRNP A1 (0.5 μg) or hnRNP C (0.1 μg) were incubated with [α-32P]Δ2R1 (8 × 10⁶ cpm), UV cross-linked, and digested with RNase as described above. RNA-protein complexes were incubated with trypsin (ranging from 0.25 to 50 ng, 200-1 ml) with 50 mM Tris-HCl pH 8.0, 320 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin A for 0,5,15, and 60 min at room temperature. Trypsin digestion was stopped by adding to SDS-PAGE sample buffer and boiling, followed by analysis under denaturing conditions by 15% SDS-PAGE.

**RESULTS**

Cytosolic Proteins with AUUUA Binding Activity (AUBP) from Resting and PHA-activated Peripheral Blood Lymphocytes—Cytoplasmic lysates were assayed for binding to in vitro transcribed, radiolabeled AU-rich sequences present in the 3'-UTR of lymphokine (IL-2, GM-CSF) or c-myc mRNAs. The Δ2R1 probe contains a sequence found in the 3'-UTR of GM-CSF c-mRNA (9) (Fig. 1). After coinubation of AUUUA-containing RNA with cytoplasmic lysates, ultraviolet cross-linking and RNase digestion were performed, the samples were then analyzed by SDS-PAGE. Three major AU-rich RNA-protein complexes (36, 43, and 50 kDa) were detected in cytoplasmic lysates from resting and activated cells (Fig. 1). The intensity of each of these bands was increased with activation. Activation also induced the appearance of two additional AUBPs (70 and 25 kDa), with the level of the 25-kDa protein being quite variable, and each usually much less relative to the other proteins. The increases in AUBP upon activation were not readily apparent before lectin activation. Similar patterns of AU-specific binding proteins were observed with purified (>90% CD4+) T cell preparations (data not shown).

In previous studies, we found that the interaction between the AUUUA-containing RNA probes and the 30-40-kDa AUBP is specifically with the reiterated AUUUA sequence (5). Unlabeled 3' GM-CSF mRNA was able to block binding of the 36-, 43-, 50-, and 70-kDa proteins to radiolabeled 3' GM-CSF mRNA probe, thus demonstrating the AUUUA
Fig. 1. 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 × 10^6 cpm of indicated ^32P-RNA probes, UV cross-linked, and RNase digested before analyzing under reducing conditions by SDS-PAGE. Lower panel, sequence of RNA probes with AUUUA-containing c-fos mRNA was selectively increased after prolonged transcriptional inhibition, the stability of AUUUA-containing c-fos mRNA was selectively increased over time (23). The effect of transcriptional inhibition upon the activity of AUBPs was therefore examined (Fig. 3). Treatment of PHA-activated lymphocytes with actinomycin D or DRB increased the level of the 36-kDa AU-specific binding protein after 30–60 min. Cycloheximide treatment alone, or

specificity of the RNA-protein interaction (Fig. 2, upper panel). The cross-reactivity and specificity of the AUBP for reiterated AUUUA sequences was investigated (Fig. 2, middle panel). Radiolabeled IL-2 3'-UTR binding to AUBP was competed by the unlabeled 3'-UTR of GM-CSF, c-myc, and itself, while the antisense of the 3'-UTR of GM-CSF did not compete at all. To further insure that the observed AUBP binding did not require the presence of additional sequences, the antisense RNA of the 3'-UTR of IL-2 mRNA (IL-PAS which contains 5 AUUUUA multimers) was examined (Fig. 2, lower panel). The binding of AUBP to the antisense of the 3'-UTR of IL-2 mRNA was competed equally well by both unlabeled 3'-UTR of GM-CSF and the antisense IL-2, further indicating the specificity of the observed binding. The size of the 36-, 43-, and 70-kDa AUBPs are similar to those that have been differentially observed in separate reports (9–13). As found previously (9), pretreatment of cytoplasmic lysates with proteinase K abolished all AUBP activity, confirming the polypeptide nature of their binding activity (data not shown).

Modulation of Cytoplasmic AUBP by Transcriptional but Not Translational Inhibition—Previous work has shown that after prolonged transcriptional inhibition, the stability of AUUUA-containing c-fos mRNA was selectively increased over time (23). The effect of transcriptional inhibition upon the activity of AUBPs was therefore examined (Fig. 3). Treatment of PHA-activated lymphocytes with actinomycin D or DRB increased the level of the 36-kDa AU-specific binding protein after 30–60 min. Cycloheximide treatment alone, or
in concert with DRB treatment failed to modulate cytoplasmic levels of AUBPs, even up to 3 h of treatment (data not shown). Smaller, but substantial increases in the binding activity of the other AUBPs were also observed with transcriptional inhibition. Earlier work by Dreyfuss demonstrated a 38-kDa protein that associated with cytoplasmic poly(A)+ mRNA following transcriptional, but not translational, inhibition (14). This protein was eventually identified as the hnRNP A1, and has recently been shown to shuttle between the nucleus and cytoplasm in HeLa cells, with increased cytoplasmic accumulation following transcriptional inhibition (16).

Identification of the Cytoplasmic 36- and 43-kDa AUBPs as HnRNPs—The selective modulation of the 36-kDa AU-specific binding protein by transcriptional inhibitors as well as the size similarity of the 36- and 43-kDa AUBP with the hnRNP A1 and hnRNP C proteins (molecular mass 41 and 43 kDa) (24, 25), suggested that some or all of these proteins were hnRNPs. This issue was addressed using monoclonal antibodies directed against the hnRNPs A1 and C proteins (14–16). Cytoplasmic lysates from activated lymphocytes were incubated with radiolabeled GM-CSF 3′-UTR mRNA, UV cross-linked, and immunoprecipitated (Fig. 4). Antibody directed against the anti-hnRNP A1 (9H10) precipitated a single radiolabeled RNA-protein complex of 36 kDa from each lysate. In contrast, antibody (4F4) which recognizes the hnRNP C1 and C2 proteins (41 and 43 kDa by SDS-PAGE) precipitated two bands (43 and 36 kDa). The relative ratio of the 43-kDa RNA-protein complex to that of the 36-kDa RNA-protein complex appeared to vary in immunoprecipitation experiments with the 4F4 antibody. In addition, 4F4 occasionally weakly precipitated a 70-kDa protein (experiment 2). These latter results are not unexpected, as previous studies have demonstrated that this anti-hnRNP C antibody (4F4) immunoprecipitates multiple hnRNPs (including hnRNP A1) in the absence of ionic detergents (15). Nevertheless, the detection of these specific immunoprecipitated protein-RNA complexes indicates their direct physical contact with the radiolabeled AUUU-containing RNA probe (26), and establishes both of these proteins as having the appropriate size and immunologic cross-reactivity with the hnRNP A1 and C proteins. Given the apparent cross-reactivity of the 4F4 antibody with hnRNP A1, we have also demonstrated that under these conditions, 9H10, but not the 4F4 or SP20 ascites, can immunoprecipitate recombinant hnRNP A1–radiolabeled 3′-UTR GM-CSF RNA complex (data not shown).

These observations suggested that the 36-kDa AUBP represented hnRNP A1. The ability of recombinant hnRNP A1 to specifically bind to sequences containing reiterated AU-rich sequences was therefore examined. Recombinant hnRNP A1 specifically bound the GM-CSF and IL-2 mRNA probes, while binding the antisense of the GM-CSF probe or the transforming growth factor-β2 mRNA (one AUUA sequence) was markedly diminished, paralleling that observed with cytoplasmic lysates (Fig. 5, upper and middle panels). This specificity was confirmed by demonstrating that unlabelled 3′-UTR GM-CSF, IL-2, or c-myc, but not the antisense of the 3′-UTR GM-CSF mRNA, could inhibit the binding of recombinant hnRNP A1 to radiolabeled 3′-UTR GM-CSF mRNA (Fig. 5, lower panel). Thus, recombinant hnRNP A1 demonstrates identical sequence specificity as the 36-kDa protein in our lymphocyte lysates.

Finally, the effects of partial proteolysis on AUBP and recombinant hnRNP A1 were compared (Fig. 6). Either recombinant hnRNP A1 or a cytoplasmic lysate was incubated with radiolabeled 3′-UTR GM-CSF mRNA, UV cross-linked, and subjected to partial proteolysis with trypsin. With 5 min of trypsin treatment, a marked reduction in the hnRNP A1–RNA complex is observed, disappearing completely after 15 min.


appearance of the 25-kDa AUBP (Fig. 7). When proteolytic digestion was performed following RNA binding, a significant diminution in the 43-kDa AUBP was not observed (77% of control after 60 min), allowing us to conclude that, under these conditions, the 25-kDa AUBP appeared to derive primarily from the 36-kDa AUBP, which we have identified as hnRNP A1. Recombinant hnRNP C was considerably more resistant to trypsin digestion than recombinant hnRNP A1 (Fig. 6, bottom), and therefore approximates what was observed for the 43-kDa AUBP in the cytoplasmic lysates. Trypsin digestion of recombinant hnRNP C-RNA complex yields 18- and 14-kDa AUBPs. These studies provide further evidence that the 25-kDa AUBP observed with partial proteolysis of the cytoplasmic lysates is generated from the 36-kDa AUBP that we have identified as hnRNP A1, and not the 43-kDa AUBP protein we have identified as hnRNP C.

DISCUSSION

These data indicate that two of the AUBPs detected in these experiments are members of the family of hnRNPs. In particular, the identification of the cytoplasmic 36-kDa protein as hnRNP A1 would seem strongly supported by our findings: (i) specific immunoprecipitation; (ii) modulation of cytoplasmic levels with transcriptional, but not translational inhibitors; (iii) comparable patterns of AU-specific RNA binding between the 36-kDa AUBP and recombinant hnRNP A1; (iv) comparable sensitivity and AUBP patterns generated by partial proteolysis. By similar, but slightly less stringent criteria (size and immunoreactivity), the cytoplasmic 43-kDa AUBP would appear to represent a member of the hnRNP C protein family, but another immunologically related hnRNP cannot be conclusively excluded due to the lack of absolute specificity of the anti-hnRNP C antibody.

The hnRNP proteins are a family of abundant nuclear proteins that has been generally implicated in mRNA metabolism at the level of pre-mRNA splicing (25, 30, 31). The hnRNP A1 and C proteins have been shown to bind to a polypyrimidine stretch bordered by AG at the 3' end of introns (30, 31), and antibodies to hnRNP C have been shown to inhibit splicing reactions in vitro (25). With the discovery that hnRNP A1 shuttles between the nucleus and cytoplasm (16), the possibility exists that hnRNP A1 may exert addi-
tional influences upon mRNA metabolism beyond that of nuclear processing. Since intron-containing pre-mRNAs are spliced prior to nuclear export, and cytoplasmic hnRNP A1 has been shown to be associated with mRNA (16), the findings reported here raise the possibility that the hnRNP A1 protein we have described may favor association with RNA polymerase II transcripts that contain reiterated AUUUUA sequences.

The possible etiology of hnRNP As as AUBPs was first raised by Vakalopoulou et al. (13), who reported 32- and 43-kDa nuclear proteins with AUBP activity. Through immunoprecipitation, this 43-kDa nuclear protein was identified as being immunologically related to an hnRNP C protein, thus supplying the first identification of an hnRNP protein's ability to bind to reiterated AUUUUA sequences. Our studies thus serve as confirmation of this original description, but differ significantly as the 43-kDa AUBP was found in the cytoplasm, and not the nucleus. Because of the above finding, these authors considered, and rejected, the notion that the 32-kDa AUBP found in the nucleus and, to a lesser extent, the cytoplasm of HeLa cells, might represent an hnRNP A protein. This conclusion was based in part, on their inability to immunoprecipitate this protein with the relevant antibody (13).

Their findings raise the issue that the identity of the cytoplasmic 32-kDa AUBP reported by this group might not be hnRNP A1, although another possibility may be that hnRNP A1 is not easily precipitated in certain cell types or subcellular sites. Indeed, we have found that under conditions that would completely immunoprecipitate 1 μg of recombinant hnRNP A1, only a fraction of the cytoplasmic 36-kDa AUBP is immunoprecipitable. A similar inability to immunodeplete with anti-hnRNP C antibody was observed (data not shown).

A second possibility is that hnRNP A1 proteins in the nucleus and cytoplasm of these two different cell types may represent differing isoforms or alternately spliced forms (hnRNP A2) of the same hnRNP A1 gene family (17, 33, 34). However, antibodies to hnRNP A1 have been shown to detect these alternate forms (33, 34), making this possibility unlikely. Third, these two proteins may be completely unrelated, as is suggested by the report that partial proteolysis of the 34-kDa nuclear AUBP from marmoset T cells was quite distinct from that of purified hnRNP A1 (38). It is unclear if these experiments involved assay of hnRNP A1 binding to AUUUUA multimers, and whether any AUUUUA binding activity of hnRNP A1 was observed.

In any case, the evidence we have presented (modulation by transcriptional inhibitors, immunologic cross-reactivity, comparable binding profiles with recombinant hnRNP A1 protein) would seem to establish that the 36-kDa cytoplasmic protein is a member of the hnRNP A1 family of proteins. Moreover, hnRNP A1 has been shown to have a tryptophan sensitive site which, when cleaved, generates a 25-kDa protein, UP-1 (27, 28), initially described in thymic extracts for its ability to bind single-stranded DNA (29). Given identification of the cytoplasmic 36-kDa AUBP as hnRNP A1, as well as the partial proteolysis experiments, the occasionally observed 25-kDa AUBP would seem likely to represent UP-1, which may occur in vivo (28). Finally, hnRNP A1 has been demonstrated in the cytoplasm of activated lymphocytes and is modulated by transcriptional inhibitors, thus confirming the cytoplasmic location observed in these experiments.2 Unresolved in these studies is the identity of the other cytoplasmic AUBPs that we have detected. Our findings suggest that the AUBPs might be either members of, or related to, the family of hnRNPs, all of which contain RNA-binding domains (36, 37). The reactivity of the 4F4 antibody with the 43- and 70-kDa AUBP would suggest that the latter protein might also be a member of the hnRNP family.

The finding that hnRNP C protein is a cytoplasmic AUBP differs from previous work (13) where hnRNP C was identified as a nuclear AUBP in HeLa cells. These studies raise the possibility that the hnRNP C detected in our experiments may represent hnRNP C leakage from the nucleus. Alternatively, the different subcellular localization of these proteins may result as a consequence of differing cell types and growth conditions (resting peripheral blood lymphocytes as opposed to an actively replicating HeLa cell line). Several lines of evidence support the hypothesis that nuclear leakage of hnRNP C does not occur under the conditions utilized in these experiments. First, Western blotting of cytoplasmic lysates did not demonstrate the 70-kDa U1 small nuclear (sn) RNP (data not shown), indicating that leakage of this nuclear protein had not occurred. Thus, nuclear contamination of our cytoplasmic lysates was therefore not evident by this index.

Second, cytoplasmic preparations of human peripheral blood monocytes are devoid of AUBP activity relative to resting lymphocytes (data not shown), indicating that nuclear leakage of hnRNP C from a cell closely related to the lymphocyte is not observed. Moreover, it indicates that the lymphocyte appears distinct from the monocyte in terms of the presence of cytoplasmic hnRNP, making our findings with those of Vakalopoulou et al. (13) less surprising. Third, previous studies have indicated that hnRNP A are tightly associated with nuclear mRNA (38), such that hnRNP leakage from the nucleus would not be expected to easily occur. In support of this view has been our finding that the profile of cytoplasmic AUBP was unchanged over 1–10 min of detergent exposure (data not shown). Moreover, using different methods of cytoplasmic lysate preparation, other groups have also observed a cytoplasmic 43-kDa AUBP (10, 11). Despite these considerations, it is clear that definitive localization of hnRNP C as a cytoplasmic protein is beyond the scope of this study. These studies identify the 43-kDa AUBP that we have found in cytoplasmic preparations of peripheral blood lymphocytes as the hnRNP C protein, which thereby raises interesting issues relative to both the cytoplasmic localization and the biologic role of hnRNP C in lymphocytes.

Given the well characterized role of the AUUUUA sequences in the regulation of cytoplasmic mRNA turnover and translation (5–8), this characterization of two hnRNPs as AUBPs leads to the consideration of a novel biologic role for the hnRNP protein family. An analysis of the functional relevance of their binding to lymphokine mRNA is beyond the scope of this article and promises to be difficult, given other studies that have sought a direct consistent correlation between AUBP binding in vitro and mRNA turnover. In a previous study (13), an AUUUUA motif flanked by polyuridines efficiently bound a 32-kDa AUPB, but mRNA lability (both nuclear and cytoplasmic) required at least three AUUUUA sequences. This finding would suggest that binding of an AUPB to the reiterated AU sequences facilitates RNA turnover, as would the report that 37- and 40-kDa c-myc-specific AUBPs exhibited intrinsic nuclear activity (39). Although we are unaware of any reports of nuclease activity being exhibited by hnRNPs, we have found that increases in cytoplasmic AUPB do not occur prior to 8–12 h of PHA activation, a time when lymphokine mRNA levels begin to decline despite continued high rates of gene transcription (40).

These data would be most consistent with the hypothesis that cytoplasmic levels of the 36-kDa AUPB/hnRNP A1 directly correspond to increased mRNA turnover. This theory is in general agreement with the observation that co-stimu-
lation of T cells with a phorbol ester and anti-CD3 antibody induces the disappearance of a 30-kDa AUBP, while increasing lymphokine mRNA stability (11). In contrast, the increase in cytoplasmic hnRNP A1 that occurred with inhibition of RNA polymerase II would suggest an mRNA stabilizing activity given the report that transcriptional inhibition reduces the turnover of mRNA containing reiterated AUUUA sequences (23). However, this effect of transcriptional inhibition requires prolonged (>2 h) treatment with DRB or actinomycin D, in contrast to the more rapid (30 min) modulation of hnRNP A1, suggesting that another mechanism might mediate this effect. It is clear that AUUUA-dependent post-transcriptional regulation of gene expression will involve multiple mechanisms, as we found that cycloheximide treatment had no effect of cytoplasmic AUBP levels although it has been reported to increase the stability of lymphokine mRNA (41, 42). The complexity of these pathways is further supported by the observation that the turnover of cytokine mRNA can be uncoupled from that of proto-oncogenes such as c-fos and c-myc (43-45).

In conclusion, these studies would suggest that hnRNP A1 and C proteins present in human T lymphocytes are capable of specifically binding to the AU-rich sequences contained in the 3′-UTR of lymphokine and c-myc mRNA. As these cis-acting sequences have been shown to be functionally important determinants in the post-transcriptional regulation of gene expression, the association of these proteins with the reiterated AUUUA sequences may play an important role in mRNA turnover and translation. With the identification of these proteins, future studies can now be more carefully directed at defining their functional role(s) in the post-transcriptional regulation of lymphokine gene expression.

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REFERENCES

1. Ross, J. (1986) Mol. Biol. Med. 3, 1–14
2. Casey, J. L., Hentze, M. W., Koeller, D. M., Caughman, S. W., Rouault, T. A., Klausner, R. D., and Harford, J. B. (1988) Science 240, 924–928
3. Mullner, E. W., and Kuhn, L. C. (1988) Cell 53, 815–825
4. Klausner, R. D., and Harford, J. B. (1989) Science 246, 870–872
5. Caput, D., Beutler, B., Hartog, K., Brown-Shimer, S., and Cerami, A. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1670–1675
6. Shaw, G., and Kammer, R. (1986) Cell 46, 659–667
7. Kruey, V., Marinz, O., Shaw, G., Deschamps, J., and Huez, G. (1989) Science 245, 855–858
8. Han, J., Brown, T., and Beutler, B. (1990) J. Exp. Med. 171, 465–475
9. Maller, J. S. (1989) Science 246, 664–666
10. Gillis, P., and Maller, J. S. (1991) J. Biol. Chem. 266, 3172–3177
11. Buvoli, M., Petrucci, B., June, C. H., Thompson, C. B., and Lindsten, T. (1991) Mol. Cell. Biol. 11, 3288–3295
12. Bohjanen, P. R., Petrucci, B., June, C. H., Thompson, C. B., and Lindsten, T. (1992) J. Biol. Chem. 267, 6302–6308
13. Vakalopoulou, E., Schack, J., and Shenk, T. (1991) Mol. Cell. Biol. 11, 3355–3364
14. Dreyfuss, G., Adam, S. A., and Choi, Y. D. (1984) Mol. Cell. Biol. 4, 415–422
15. Pinol-Roma, S., Choi, Y. D., Matunis, M. J., and Dreyfuss, G. (1988) Genes & Dev. 2, 215–227
16. Pinol-Roma, S., and Dreyfuss, G. (1989) Nature 335, 702–703
17. Buvoli, M., Bismonti, G., Tocilfis, P., Basili, M. T., Berti, A., Riva, S., and Morandi, C. (1988) Nucleic Acids Res. 16, 3751–3770
18. Nakagawa, T. Y., Swanson, M. S., Wold, B. J., and Dreyfuss, G. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 2007–2011
19. Cervera, M., Dreyfuss, G., and Pennanen, S. (1981) Cell 23, 113–120
20. Holbrook, N. J., Smith, K. A., Furnace, A. J., Comeau, C. M., Wisicok, R. L., and Crabtree, G. R. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 1634–1638
21. Altitalo, K., Schwab, M., Lin, C. C., Varumis, H. E., and Bishop, J. M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 1707–1711
22. de Martin, K., Hainzeller, B., Hofer-Warkine, R., Guigasch, H., Wraen, M., Schliessner, H., Seifert, J. M., Bodmer, S., Fontana, A., and Hofer, E. (1987) EMBO J. 6, 3673–3677
23. Slova, A. B., Greenberg, M. E., and Belasco, J. G. (1989) Genes & Dev. 3, 60–72
24. Beyer, A. L., Christensen, M. E., Walker, B. W., and LeStouregeon, W. M. (1977) Cell 11, 127–138
25. Choi, Y. D., Grubowski, P. J., Sharp, P. A., and Dreyfuss, G. (1986) Science 231, 1354–1359
26. Pinol-Roma, S., Adam, S. A., Choi, Y. D., and Dreyfuss, G. (1989) Methods Enzymol. 180, 410–424
27. Riva, S., Morandi, C., Tocilfis, P., Pandolfo, M., Bismonti, G., Merrill, B., Williams, K. R., Multhaup, G., Beyreuther, K., Werr, H., Henrich, B., and Schliess, J. (1986) EMBO J. 5, 2267–2273
28. Pandolfo, M., Valenti, O., Bismonti, G., Morandi, C., and Riva, S. (1985) Nucleic Acids Res. 13, 6577–6580
29. Herrera, G., and Alberts, B. (1976) J. Biol. Chem. 251, 2124–2132
30. Mayerda, A., and Krainer, A. R. (1992) Cell 69, 355–375
31. Swanson, M. S., and Dreyfuss, G. (1989) EMBO J. 7, 3519–3529
32. Buvoli, M., Colbieni, F., Bismonti, G., and Riva, S. (1990) Nucleic Acids Res. 18, 6985–6990
33. Buvoli, M., Colbieni, F., Bertagno, M. G., Mangiarotti, A., Bassi, M. T., Bismonti, G., and Riva, S. (1990) EMBO J. 9, 1229–1235
34. Battaglia, Colbieni, F., Bertagno, M., Bassi, M. T., Bismonti, G., and Riva, S. (1990) Mol. Cell. Biol. 14, 83–84
35. Myer, V. E., Lee, S. I., and Steitz, J. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1299–1303
36. Dreyfuss, G., Philipson, L., and Mattaj, I. W. (1988) J. Cell Biol. 106, 1419–1429
37. Bord, C. G., Swanson, M. S., Gorlach, M., and Dreyfuss, G. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9788–9792
38. Dreyfuss, G. (1986) Annu. Rev. Cell. Biol. 2, 459–466
39. Brewer, G. (1991) Mol. Cell. Biol. 11, 2493–2496
40. Kronek, M., Leonard, W. J., Depper, J. M., and Greene, W. C. (1985) J. Cell Biol. 105, 1250–1258
41. Wiltson, T., and Treisman, R. (1988) Nature 338, 396–399
42. Pearson, R., Elton, T., Nissen, M. S., Lehn, D., and Johnson, K. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6333–6335
43. Schuler, G. D., and Cole, M. D. (1986) Cell 55, 1115–1122
44. Lindsten, T., June, C. H., Ledbetter, J. A., Stella, G., and Thompson, C. B. (1989) Science 244, 333–343
45. Bickel, M., Cohen, R. H., and Pluznik, D. H. (1990) J. Immunol. 145, 840–845