AU-A, an RNA-binding activity distinct from hnRNP A1, is selective for AUUUA repeats and shuttles between the nucleus and the cytoplasm

David A.Katz, Nicholas G.Theodorakis¹, Don W.Cleveland¹, Tullia Lindsten and Craig B.Thompson*

Departments of Molecular Genetics and Cell Biology, and Medicine, and Howard Hughes Medical Institute, University of Chicago, 5841 S. Maryland, MC 1028, Chicago, IL 60637 and ¹Department of Biological Chemistry, Johns Hopkins University, School of Medicine, Baltimore, MD 21205, USA

Received August 9, 1993; Revised and Accepted December 6, 1993

ABSTRACT

The 3'-untranslated regions of many labile transcripts contain AU-rich sequences that serve as *cis* determinants of mRNA stability and translational efficiency. Using a photocrosslinking technique, our laboratory has previously defined three cytoplasmic RNA-binding activities specific for the AUUUA multimers found in the 3'-untranslated regions of lymphokine mRNAs. One of these activities, AU-A, has an apparent molecular mass of 34 kDa, is constitutively expressed in both primary T cells and the Jurkat T cell leukemia line, and binds to a variety of U-rich RNA sequences. Previous studies had shown that AU-A is more prevalent in the nucleus than the cytoplasm, raising the possibility that AU-A is really a nuclear RNA-binding activity that is found in cytoplasmic extracts because of nuclear leakage during cell fractionation. We now show that AU-A shuttles between the cytoplasm and the nucleus. Our results indicate that AU-A is a candidate protein component of ribonucleoprotein complexes that participate in nucleocytoplasmic transport of mRNA and cytoplasmic mRNA metabolism. The properties of AU-A activity are similar to those of heterogenous nuclear ribonucleoprotein A1 (hnRNP A1). However, using monoclonal antibodies to hnRNP A1 and protease digestion patterns, we show that AU-A activity and hnRNP A1 protein are distinct. These studies have also allowed us to define a fourth RNA-binding activity of apparent molecular mass 41 kDa with specificity for AUUUA multimers. This activity is restricted to the nucleus and contains the hnRNP C protein.

INTRODUCTION

The functional expression of a eukaryotic protein from DNA involves several distinct processes. Expression of many genes is regulated by the rate of transcription of DNA to RNA.

However, changes in the functional level of a protein do not always correlate with the transcription rate, indicating that control of expression also occurs posttranscriptionally. Targets for posttranscriptional regulation of gene expression include nuclear RNA processing, RNA export from the nucleus, cytoplasmic mRNA stability, and translational efficiency. The 3' untranslated regions (UTRs) of many short-lived mRNAs, including those that encode lymphokines and proto-oncogenes, contain conserved AU-rich sequences (1). A number of studies have demonstrated that these sequences are *cis* determinants of cytoplasmic mRNA stability. Insertion of AU-rich sequences from the UTRs of either GM-CSF or *c-fos* mRNAs into the UTRs of genes with normally stable mRNAs leads to decreased mRNA stability (2–5). Conversely, deletion or mutation of these AU-rich regions from the UTRs of short-lived genes has been shown to confer stability on their mRNAs (3, 6, 7).

The dependence of AU-rich region-mediated mRNA instability on translation was first demonstrated by pharmacological inhibition of translation (6). In the presence of cycloheximide, the normally labile *c-fos* mRNA was significantly stabilized. Translational inhibition blocks mRNA deadenylation, which is apparently the first step of mRNA degradation directed by AU-rich regions (6, 8). A further indication of the link between translation and mRNA instability was the observation that while the AU-rich region of GM-CSF mRNA directed translation-dependent mRNA degradation when placed in the 3'UTR of β -globin mRNA, the same sequence did not so function when placed within the coding region of β -globin mRNA (9). These authors suggested that mRNA degradation is mediated by a large complex they observed associated with labile mRNAs. Formation of this complex required both AU-rich regions and active translation, but was blocked by ribosome translocation across an AU-rich sequence placed in a protein-coding region. Treatment of cells with either actinomycin D or DRB increased stability of a β -globin mRNA containing the AU-rich region of the *c-fos* mRNA (5). This observation suggests that AU-rich region-mediated mRNA stability may also depend on ongoing transcription.

* To whom correspondence should be addressed

AU-rich regions in the 3' UTRs of labile mRNAs have also been shown to be *cis* determinants of translation rates. Blockade of lymphokine or proto-oncogene translation was demonstrated in *Xenopus* oocytes, in which the normal mRNA degradation pathway does not occur (10, 11). This blockade depended on the AU-rich regions in 3' UTRs; no effect was observed when the AU-rich regions were moved to 5' UTRs of the same mRNAs (12). In macrophages, TNF- α translation is upregulated in response to specific inducing signals; this effect is dependent on AU-rich regions in the 3' UTR of TNF- α mRNA as demonstrated by studying translation of reporter constructs containing various portions of 5' and 3' TNF- α UTRs (13, 14).

Using regulation of transcription by sequence-specific DNA-binding proteins as a model, it seems reasonable to propose that RNA-binding proteins specific for AU-rich regions are *trans* determinants of cytoplasmic mRNA metabolism. Previous work demonstrated the presence in purified human T cells of three cytoplasmic activities (AU-A, AU-B, AU-C) that bind to the AUUUA multimers present in lymphokine mRNAs (15, 16). AU-A is a 34 kDa protein (or complex) constitutively expressed in peripheral T lymphocytes and the Jurkat T cell leukemia line. AU-A is more prevalent in the nucleus than the cytoplasm. AU-A binds to several other U-rich RNA sequences, including the AU-rich region of *c-myc* mRNA, and poly-U (16). Several other groups have also observed RNA-binding activities similar to AU-A in extracts of human cell lines. AUBF is a 36 kDa factor present in cytoplasmic extracts of Jurkat cells that interacts with the AU-rich sequences of both lymphokine and proto-oncogene mRNAs (17). A 32 kDa factor with similar binding specificity

has been observed in nuclear extracts of HeLa cells (4), a 33 kDa RNA-binding factor with specificity for the AU-rich region of GM-CSF 3' UTR was recently observed in cell extracts derived from mouse, rat and human (18), and a 35 kDa factor that binds to β -adrenergic receptor mRNAs was recently described (19).

One interesting property of AU-A is its presence in both nucleus and cytoplasm (15). We have now studied the subcellular distribution of AU-A in Jurkat cells in greater detail. Our experiments showed that AU-A shuttles between nuclear and cytoplasmic complexes, and AU-A redistributes to cytoplasm in the absence of ongoing RNA polymerase II transcription. It has previously been shown that a subset of the heterogenous nuclear ribonucleoproteins (hnRNPs) shuttle in a similar fashion between nucleus and cytoplasm (20). Based on similar properties, we investigated the possibility that one of these proteins, hnRNP A1, is a constituent of AU-A activity. Using monoclonal antibodies specific for hnRNP A1, and protease digestion patterns, we demonstrate that cytoplasmic AU-A activity is distinct from hnRNP A1 protein. We also observed an approximately 41 kDa RNA-binding activity with specificity for AU-rich sequences. This activity is restricted to the nucleus and was found to contain hnRNP C protein. Our results suggest that AU-A may be part of a complex that is a substrate of nucleocytoplasmic transport or cytoplasmic metabolism of mRNA. We propose that exchange of other RNA-binding proteins for AU-A on AUUUA sequences in the cytoplasm could be involved in the regulation of mRNA degradation.

MATERIALS AND METHODS

Cell culture

Jurkat cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin G and 100 μ g/ml streptomycin to a density of approximately 10^6 /ml for all experiments. For inhibition of transcription, Jurkat cells were treated with 5 μ g/ml actinomycin D or 100 μ M 5,6-dichloro- β -d-ribofuranosyl benzimidazole (DRB) for 3 hours. For inhibition of translation, Jurkat cells were treated with 20 μ g/ml cycloheximide for 3 or 4 hours. Cells were counted using a Coulter Counter apparatus immediately before fractionation. K562 cells were split 1:12 two days prior to use for sucrose gradient analysis of cytoplasm.

Cell fractionation

All steps were carried out at 4°C. Cytoplasm/nucleus separation and cytoplasmic fractionation were performed essentially as described by Brewer and Ross (21). Cells were harvested by centrifugation for 2 minutes at $250\times g$, then washed twice with phosphate-buffered saline. For cytoplasm/nucleus separation, approximately 10^8 cells were resuspended in 3.5 ml of a hypotonic buffer containing 10 mM Tris pH 7.4, 5 mM magnesium chloride, 1.5 mM potassium acetate, 2 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride. Cells were disrupted in a Dounce homogenizer using 20 strokes of an A pestle. Nuclei were harvested by centrifugation for 5 minutes at $250\times g$. Polysomes and other supramolecular complexes were pelleted by centrifugation of the cytoplasmic supernatant through a cushion of 30% sucrose in the above buffer for 75 minutes at $130000\times g$. S130 is the supernatant from this step, excluding the cloudy material found at the interface between S130 and the sucrose pad, which was collected separately. The polysome-

Figure 1. AU-A is associated with large cytoplasmic complexes and found in the nucleoplasm. Proteins from subcellular fractions of 10^6 Jurkat cells (except N, for which protein from nucleoplasm of 10^5 Jurkat cells was used) were crosslinked to [^{32} P] UA(UUUUA)₅CUCG, separated electrophoretically in a 10% SDS-polyacrylamide gel and detected by autoradiography. Lane 1 = S130 (cytosol); lane 2 = S130/sucrose pad interface (microsomal fraction); lane 3 = ribosomal salt wash; lane 4 = washed polysomes; lane 5 = nuclear membrane; lane 6 = nucleoplasm; lane 7 = nucleoli + chromatin.

containing pellet was washed and resuspended in 0.5 ml of the above hypotonic buffer, then adjusted to 0.3 M potassium chloride by slow addition of 4 M potassium chloride. Salt-washed polysomes were pelleted by centrifugation through a cushion of 30% sucrose as above. Ribosomal salt wash is the supernatant from this step. Nuclear fractionation was performed essentially as described by Beebe (22). The nuclear pellet from above was washed in 2.5 ml of 10 mM Tris pH 7.4, 5 mM magnesium chloride, 0.25 M sucrose, 0.5% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride to remove the outer nuclear membrane. Nuclei were pelleted by centrifugation for 5 minutes at $250\times g$, then resuspended in 2.5 ml of the above buffer without magnesium chloride or Triton X-100, and sonicated 4×10 seconds. Nucleoli and insoluble chromatin were pelleted by centrifugation of this sonicate through a cushion of 0.88 M sucrose for 20 minutes at $2000\times g$. The supernatant from this step is the nucleoplasm. Protein concentration was quantitated using the BioRad Protein Assay.

Sucrose gradient analysis of cytoplasm

All steps were carried out at 4°C . Cells were washed twice with phosphate-buffered saline containing 100 $\mu\text{g}/\text{ml}$ cycloheximide. For cytoplasm/nucleus separation, approximately 6×10^6 cells were resuspended in 1.0 ml of 0.5% NP-40 lysis buffer containing 10 mM HEPES pH 7.4, 10 mM potassium chloride, 5 mM magnesium chloride and 100 $\mu\text{g}/\text{ml}$ cycloheximide. Nuclei were pelleted by centrifugation for 1 minute at $12000\times g$. Cytoplasm was adjusted to 100 mM potassium chloride, and to 20 mM EDTA where indicated. The resulting supernatant was layered on a linear 10–50% (w/v) sucrose gradient in the same buffer, and gradients were centrifuged for 2 hours at $130000\times g$. Gradient fractions were collected with an ISCO model 185 density gradient fractionator connected to a type 6 optical unit and UA5 absorbance monitor.

RNA-binding assay

Protein extracts or purified proteins were incubated at room temperature for 30 minutes with 10^5 cpm of [^{32}P] RNA (15) in a buffer containing 10 mM HEPES pH 7.6, 40 mM potassium chloride, 3 mM magnesium chloride, 1 mM dithiothreitol and 5% glycerol. Protein–RNA complexes were incubated at room temperature for 10 minutes with 1 unit/ μl RNase T1 (Calbiochem), then at room temperature for 10 minutes with 5 $\mu\text{g}/\mu\text{l}$ heparan sulfate to reduce nonspecific binding. The sequence of the major RNase T1 fragment of the [^{32}P] RNA used is UA(UUUA)₅CUCG. RNA was crosslinked to bound proteins by 25–40 μJ of 254 nm UV radiation on ice using a Stratilinker apparatus (Stratagene). RNA–protein complexes separated by SDS-polyacrylamide gel electrophoresis were detected by autoradiography on Kodak XAR film, and quantitated using a PhosphorImager apparatus (Molecular Dynamics). Averages, standard deviations and correlation coefficients were calculated using Excel 2.2 software (Microsoft).

Immunoprecipitation

Immunoprecipitations were performed essentially as described by Choi and Dreyfuss (23). All steps were carried out at 4°C . 1–5 μg monoclonal antibody was incubated for 60 minutes with formalin-fixed *Staphylococcus aureus* cells (Pansorbin, Calbiochem) in phosphate-buffered saline containing 1% Empigen BB (Calbiochem), 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, and

0.1 mg/ml aprotinin. Immunoprecipitates were formed by 60 minute incubation of crosslinked RNA–protein complexes with the Pansorbin-antibody pellet in the same buffer to which 2 units/reaction RNasin (Promega) had been added. Immunoprecipitates were washed twice in the above buffer, then denatured by boiling in 2% SDS/2% β -mercaptoethanol. In some experiments, crosslinked RNA–protein complexes were preincubated with Pansorbin to reduce non-specific binding. Monoclonal antibodies 7A9 (specific for hnRNPs A, B, E, G, H, and L), 4B10 (specific for hnRNP A1) and 4F4 (specific for hnRNP C) (24) were generated from the SP2/0 myeloma.

Immunoblotting

Immunoblots were performed using a 1:500 dilution of affinity purified monoclonal antibody, followed by a 1:3000 dilution of Alkaline Phosphatase/Goat anti-Mouse IgG Conjugate (Jackson Laboratories) in tris-buffered saline containing 4% bovine serum albumin. Immunoblots were developed using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (BioRad).

RESULTS

AU-A activity is found primarily in large cytoplasmic complexes and nucleoplasm

We were interested to determine the function and nature of a set of cytoplasmic RNA-binding activities previously described to bind to AUUUA multimers (15, 16). The observation that greater than 95% of total cellular AU-A is nuclear raised the possibility that it was present in the cytoplasm as a result of nuclear leakage during cell fractionation. Therefore, the subcytoplasmic and subnuclear distribution of AU-A was studied by fractionation of cells from the human T cell leukemia line Jurkat (figure 1). The cytoplasmic pool of AU-A was found predominantly in a subcytoplasmic fraction that contains less than 5% of total cytoplasmic protein (lane 3). This fraction, the ribosomal salt wash, is known to contain a number of proteins associated with polysomes, but which are not primary constituents of the ribosomal subunits. It also may contain proteins extracted by salt from other supramolecular cytoplasmic complexes. The proteins found in the ribosomal salt wash include translation initiation factors (25), an exonuclease associated with mRNA degradation (26, 27), and proteins that bind to the 3' UTRs of oncogene and histone mRNAs (28, 29). Very little or no AU-A activity was observed in the cytosol (lane 1), a membrane fraction (lane 2) or washed polysomes (lane 4).

In the nucleus, AU-A activity was found mostly in the nucleoplasm (lane 6), which contains about 40% of nuclear protein. Little AU-A was detectable in the outer nuclear membrane (lane 5), or a fraction containing nucleoli and insoluble chromatin (lane 7). Based on the method of preparation, we expect that many nuclear RNA-binding proteins, including those of hnRNP and U snRNP particles, are contained in the nucleoplasm (30, 31). We also noted a 41 kDa (after subtracting the mass of crosslinked RNA) nuclear RNA-binding activity specific for AUUUA multimers that also localizes to the nucleoplasm.

To further characterize cytoplasmic complexes containing AU-A, we analyzed the distribution of this activity in sucrose gradients (figure 2). Approximately 70% of the total AU-A activity detected on the gradient was found in a peak that cosedimented with 60S ribosomal subunits and 80S ribosomes (left panels, fractions 4–7). Approximately 10% of the total activity cosedimented with

Figure 2. AU-A cosediments predominantly with 60S ribosomal subunits and 80S ribosomes. Cytoplasmic extracts were prepared from K562 cells and sedimented on sucrose gradients to analyze the distribution of AU-A within polysomes or other cytoplasmic complexes. The absorbance at 260 nm was monitored during collection; 16 fractions of 0.75 ml each were collected from each gradient. Protein from each fraction was crosslinked to [^{32} P] UA(UUUA) $_5$ CUCG, separated electrophoretically in a 10% SDS-polyacrylamide gel and detected by autoradiography. The top panels show the distribution of RNA-binding activities in gradient fractions. The positions of ribosomal subunits and polysomes in the gradients are indicated. The bottom panels show the absorbance profile from each gradient. The direction of sedimentation is from left to right.

polysomes (left panels, fractions 8–15). Treatment with 20 mM EDTA to disrupt polysomes prior to gradient separation did not appreciably alter the sedimentation of the AU-A activity peak (right panels). Approximately 97% of detected AU-A activity remained at the top of the gradient when cytoplasm was treated with 300 mM potassium chloride prior to gradient separation (data not shown). This result confirmed the salt sensitivity of cytoplasmic complexes containing AU-A.

AU-A shuttles between the nucleus and the cytoplasm

The association of AU-A with a supramolecular complex in the cytoplasm suggested that this activity might be a *trans*-determinant of AUUUA-mediated control of cytoplasmic mRNA metabolism. Previous studies had demonstrated that AUUUA-mediated mRNA instability was sensitive to inhibition of cellular translation or transcription (5, 6). Thus, we investigated whether inhibitors of translation or transcription would modulate cytoplasmic levels

or localization of AU-A. Actinomycin D inhibits transcriptional elongation by intercalation into the DNA substrate. Treatment of Jurkat cells with this drug for three hours reproducibly led to an at least three-fold accumulation of cytoplasmic AU-A activity (figure 3, lanes 3 and 8). Further, cytoplasmic accumulation of AU-A did not represent accumulation of newly translated AU-A protein, since the effect of transcription inhibition was not altered by addition of cycloheximide to inhibit translation either 1 hour before (lanes 8 and 13) or simultaneously with actinomycin D (data not shown). Cytoplasmic levels of AU-A were unaffected by cycloheximide alone (data not shown). Treatment with actinomycin D, or both actinomycin D and cycloheximide, did not lead to accumulation of AU-A in the cytosol (lanes 1, 6, 11) or a microsomal fraction (lanes 2, 7, 12).

A lower level of AU-A in the nucleoplasm of Jurkat cells treated with both cycloheximide and actinomycin is likely due to normal AU-A turnover in the absence of new AU-A synthesis

Figure 3. Inhibition of RNA polymerase II by actinomycin D leads to cytoplasmic accumulation of AU-A. Proteins from subcellular fractions of 10^6 Jurkat cells (except N, for which protein from nuclei of 10^5 Jurkat cells was used) were crosslinked to [^{32} P] UA(UUUA)₅CUCG, separated electrophoretically in a 10% SDS-polyacrylamide gel and detected by autoradiography. Jurkat cells were fractionated after treatment with no drugs (lanes 1–5), actinomycin D (lanes 6–10), or cycloheximide followed by both drugs (lanes 11–15). In this figure, cell fractions are labelled as: S = S130 (cytosol), I = S130/sucrose pad interface (microsomal fraction), P = polysomes + other supramolecular complexes, M = outer nuclear membrane, N = nucleoplasm + nucleoli + chromatin. Percentages of total cellular AU-A and 41 kDa activities were calculated by equalizing all fractions for cell number (lanes N represent 1/10 the cell number of other lanes). Percentages do not add to 100% because data for lanes S, I and M were omitted for clarity of presentation.

(lanes 5 and 15). This phenomenon was also observed in Jurkat cells treated with cycloheximide alone (data not shown). In contrast to AU-A, the 41 kDa activity remained restricted to the nucleoplasm following treatment of Jurkat cells with actinomycin D, cycloheximide, or both drugs (lanes 10, 15, data not shown). Treatment of Jurkat cells with actinomycin D led to a small accumulation of AU-A in the nuclear membrane fraction (lanes 4 and 9). However, the levels of this phenomenon were not consistent between experiments.

Actinomycin D is also known to inhibit DNA replication and may have other uncharacterized activities. To confirm that the effect of actinomycin D on cytoplasmic levels of AU-A was due to transcriptional inhibition, we studied the subcellular distribution of AU-A following treatment of Jurkat cells with DRB. This drug is structurally unrelated to actinomycin D; it functions by binding to the nucleotide binding site of RNA polymerase II. Similar to actinomycin D, DRB treatment led to an at least two-fold accumulation of AU-A in the cytoplasm (figure 4, lanes 3 and 8). DRB treatment did not have any effect on the distribution of AU-A to other cytoplasmic fractions (lanes 1, 2, 6, 7) or the nuclear membrane (lanes 4 and 9). The 41 kDa activity remained restricted to the nucleoplasm following treatment of Jurkat cells with DRB (lane 10).

Cytoplasmic accumulation of AU-A could be explained either by movement of nuclear AU-A to the cytoplasm, or by an increase in the RNA-binding capacity of the cytoplasmic pool of AU-A. Because the cytoplasmic level of AU-A, even after actinomycin D treatment of Jurkat cells, was very low compared to the nuclear level of AU-A, we could not distinguish between these possibilities from the above experiments. Therefore, we studied the subcellular distribution of AU-A in Jurkat cells treated with DRB at different temperatures. If AU-A accumulation were due to modulation of RNA-binding capacity by posttranslational modification, we would expect the effect to be relatively insensitive to temperature shift during DRB treatment of cells. However, active transport across the nuclear envelope is blocked at 4°C (32). Although the total protein level (on a per cell basis) was significantly lower in cells treated for three hours with DRB at 4°C (figure 4, lanes 11–15) than in control cells cultured at 37°C without DRB (lanes 1–5), the ratio of AU-A in cytoplasm to AU-A in nucleoplasm was virtually identical (lanes 3 and 13).

This experiment was consistent with an hypothesis that transcriptional blockade enhanced transport of AU-A from nucleus to cytoplasm; however, it was also possible that cytoplasmic accumulation was due to inhibition of AU-A transport from cytoplasm to nucleus. To differentiate between these possibilities, we studied the subcellular distribution of AU-A in Jurkat cells that had been cultured with DRB at 37°C for three hours, and subsequently in fresh DRB-free medium at different temperatures (figure 4, lanes 16–25). If AU-A accumulation were due to inhibition of cytoplasm to nucleus transport, we would expect that accumulation to be reversed in cells cultured at 37°C following removal of transcriptional inhibitor from the culture medium. Under these conditions, the cytoplasmic level of AU-A was similar to or below the level observed in control cells (lanes 3 and 18). In similar experiments in which transcriptional release was done at 4°C, increased cytoplasmic levels of AU-A were maintained (lanes 8 and 23). Thus, active transport across the nuclear envelope is necessary for AU-A to move from cytoplasm to nucleus.

AU-A activity is distinct from hnRNP A1 protein; the 41 kDa activity contains hnRNP C protein

One of the few identified RNA-binding proteins shown to be present in both cytoplasm and nucleus, like AU-A activity, is the hnRNP A1 protein. The behavior of this protein in response to metabolic inhibitors and temperature shifts was similar to that of AU-A activity (20). Further, hnRNP A1 and AU-A resemble each other by the criteria of size, constitutive expression in multiple cell types, and ability to bind a broad range of U-rich RNAs. To determine whether a relationship existed between AU-A activity and hnRNP A1, we used monoclonal antibodies specific for hnRNP A1 to immunoprecipitate RNA-binding activities from Jurkat nucleoplasm (figure 5). Neither AU-A nor the 41 kDa activity were immunoprecipitated by *S. aureus* cells alone (lane 5) or by culture supernatant from the myeloma parent of the monoclonal antibodies we used (data not shown). Two RNA-binding activities were immunoprecipitated by antibodies specific for several hnRNP proteins (lane 6) or for hnRNP A1 only (lane 7). However, neither precipitated activity comigrated precisely with AU-A. Further, AU-A was not significantly depleted from nucleoplasm by immunoprecipitation using two monoclonal antibodies which have specificity for hnRNP A1 (lanes 2 and 3). Finally, we have been unable to immunoprecipitate any RNA-binding activity from Jurkat cytoplasmic fractions

Figure 4. AU-A shuttles across the nuclear envelope. Proteins from subcellular fractions of 10^6 Jurkat cells (except N, for which protein from nuclei of 10^5 Jurkat cells was used) were crosslinked to [^{32} P] UA(UUUA)₅CUCG, separated electrophoretically in a 10% SDS-polyacrylamide gel and detected by autoradiography. Jurkat cells were fractionated after treatment with no drugs (lanes 1–5) or with DRB at either 37°C (lanes 6–10) or 4°C (lanes 11–15). Additionally, Jurkat cells were fractionated after treatment with DRB at 37°C followed by 3 hours of culture in DRB-free medium at 37°C (lanes 16–20) or at 4°C (lanes 21–25). Cell fractions and percentages are as described for figure 3.

using monoclonal antibodies specific for hnRNP A1 (data not shown).

It has been reported that a monoclonal antibody specific for the hnRNP C protein immunoprecipitated a nuclear AUUUA-binding activity (4). hnRNP C resembles our 41 kDa activity by the criteria of size and nuclear restriction (20). We confirmed that the 41 kDa activity contains hnRNP C by immunoprecipitation with an hnRNP C-specific monoclonal antibody (lane 8). In contrast to our results with antibodies specific for hnRNP A1, immunoprecipitation using the 4F4 monoclonal antibody specifically and quantitatively depleted the 41 kDa RNA-binding activity from nucleoplasm (lane 4).

To provide independent evidence that cytoplasmic AU-A activity is distinct from hnRNP A1 protein, we compared the structure of the RNA-binding domains of AU-A and hnRNP A1. We used radiolabelled RNA, crosslinked to protein, as a tag for protease digestion products containing an RNA-binding domain. Following electrophoretic separation, these fragments were detected by autoradiography (figure 6). As a source of AU-A, we selected a Jurkat ribosomal salt wash preparation that was free of hnRNP C (similar to figure 1, lane 3). AU-A activity from this preparation and purified recombinant hnRNP A1 (D.Portman and G.Dreyfuss, unpublished results) have slightly different mobility in 15% polyacrylamide gels, similar to the difference in mobility between AU-A and the RNA-binding activity immunoprecipitated by hnRNP A1-specific antibodies. Further, the proteolytic products containing the RNA-binding domains of hnRNP A1 and AU-A are distinct. The cleavage patterns obtained using GluC (V8) and ArgC proteases are similar, but not identical. For example, a 21 kDa protein–RNA

Figure 5. Immunoprecipitation of RNA-binding activities by monoclonal antibodies that recognize hnRNP A1 and hnRNP C. Jurkat nucleoplasm proteins crosslinked to [^{32} P] UA(UUUA)₅CUCG were immunoprecipitated by the indicated antibodies, separated electrophoretically in a 10% SDS-polyacrylamide gel and detected by autoradiography. 7A9 recognizes hnRNP A, B, E, G, H, and L proteins. 4B10 is a monoclonal antibody specific for hnRNP A1 and 4F4 is a monoclonal antibody specific for hnRNP C. One-eighth of each supernatant, or all of each immunoprecipitate, was used in the indicated lanes.

Figure 6. AU-A and hnRNP A1 have distinct protease digestion patterns. Cytoplasmic AU-A (lanes R) or 0.4 µg purified recombinant hnRNP A1 (lanes A1) crosslinked to [^{32} P] UA(UUUA)₃CUCG were incubated for 3 hours at 37°C with the indicated amounts of GluC, ArgC or LysC proteases (Calbiochem), or without addition of any enzyme. Ribosomal salt wash isolated from 10⁶ Jurkat cells was used as the source of AU-A activity. Protease fragments containing the RNA-binding domain were detected by autoradiography following electrophoretic separation in a 15% SDS-polyacrylamide gel.

adduct that is a product of GluC digestion of hnRNP A1 was not observed in GluC digestions of AU-A.

The most dramatic difference between AU-A and hnRNP A1 was observed using LysC protease. While the RNA-binding domain of AU-A is contained within several digestion products, the hnRNP A1–RNA complex is apparently not cleaved by LysC protease. To confirm that the preparation containing the hnRNP A1–RNA adduct did not contain an inhibitor of LysC protease, we performed an immunoblot of this sample using a monoclonal antibody specific for hnRNP A1. The 4B10 epitope, which is contained within the C terminal region of the protein (S. Piñol-Roma, personal communication), was present in LysC cleavage products of 33, 25.5 and 21 kDa (data not shown). This pattern is consistent with cleavage after lysines 15, 78 and 130 of the hnRNP A1 protein. We have also noted distinct cleavage patterns of AU-A and hnRNP A1 using N-chlorosuccinimide, a chemical that cleaves after tryptophan residues (data not shown) (33).

Immunoblot analysis of the sucrose gradients shown in figure 2 revealed that cytoplasmic hnRNP A1 protein has different sedimentation characteristics than AU-A activity (figure 7). hnRNP A1 remains mostly in the top three gradient fractions, while AU-A was found predominantly in supramolecular complexes that cosediment with 60S ribosomal subunits and 80S ribosomes. This experiment also revealed that not all isoforms of hnRNP A1 are present in cytoplasmic extracts (compare lane N to all other lanes).

Figure 7. hnRNP A1 sediments principally at the top of sucrose gradients. Cytoplasmic extracts were prepared from K562 cells and sedimented on sucrose gradients to analyze the distribution of hnRNP A1 within polysomes or other cytoplasmic complexes. Gradient fractions were analyzed by immunoblotting using the monoclonal antibody 4B10. The positions of ribosomal subunits and polysomes in the gradients are indicated. The direction of sedimentation is from left to right. The gradient is the same shown in figure 2, left panels. Lane R is protein from ribosomal salt wash of 10⁶ actinomycin D-treated Jurkat cells; lane N is protein from nucleoplasm of 10⁵ actinomycin D-treated Jurkat cells.

DISCUSSION

Until recently, it was widely believed that nucleocytoplasmic export of processed RNA polymerase II transcripts was accompanied by a complete exchange of nuclear hnRNP proteins for cytoplasmic mRNA-binding proteins. Two recent experimental approaches suggest that some hnRNP proteins remain bound to RNA during export and are constituents of emergent mRNP complexes. Electron microscope tomography of a specific hnRNP complex, the Balbiani ring structure of *Chironomus tentans*, revealed that during export through the nuclear pore only some of the RNA-associated proteins were removed; others remained bound to the RNA as it emerged in the cytoplasm (34). However, the exact relationship of Balbiani ring proteins to hnRNP proteins from human cell lines has not been established. A second line of evidence suggests that some human hnRNP proteins are bound to mRNA in the cytoplasm, but such complexes have not been detected in unperturbed interphase cells. During mitosis, hnRNP proteins distribute throughout the cell following breakdown of the nuclear membrane. hnRNP proteins reaccumulate in daughter nuclei by two kinetically distinct pathways. hnRNP A1 returns to the nucleus more slowly than hnRNP C, and its return is blocked by inhibition of RNA polymerase II transcription (35). This distinction between hnRNP A1 and C also occurs during interphase. hnRNP A1 accumulation in the cytoplasm of interphase cells treated with transcriptional inhibitors was detected by immunofluorescence and photocrosslinking to polyadenylated RNA. Further, the cytoplasmic presence of hnRNP A1 in unperturbed interphase cells was inferred by the appearance of

the human protein in the *Xenopus* nucleus of an interspecific heterokaryon (20).

We had previously observed an RNA-binding activity, AU-A, which shares several properties with hnRNP A1. AU-A activity and hnRNP A1 protein are both constitutively expressed in either primary cells or cultured cell lines; both are abundant in the nucleus, but also present in the cytoplasm; both bind to a broad range of U-rich RNA sequences; and the presumed size of AU-A is similar to the known size of hnRNP A1 (15, 16, 20, 36). We have determined, however, that hnRNP A1 and AU-A are distinct by failure to immunoprecipitate AU-A with monoclonal antibodies specific for hnRNP A1, and also by demonstration that the RNA-binding domains of AU-A and hnRNP A1 are contained within distinct protease digestion fragments. Protease cleavage fragments characteristic of hnRNP A1-RNA adducts were also not detected in digestions of ribosomal salt wash from actinomycin D-treated Jurkat cells (data not shown). Additionally, hnRNP A1, as detected by immunoblotting using the monoclonal antibody 4B10, was distributed differently than AU-A activity in a sucrose gradient. Thus, AU-A is a distinct candidate constituent of ribonucleoprotein complexes that are substrates of nucleocytoplasmic transport and cytoplasmic metabolism of mRNA. We are presently investigating the possibility that AU-A may contain one of the other hnRNP proteins observed in cytoplasmic fractions of Jurkat cells (D.A.K. and S. Piñol-Roma, unpublished observations).

In this report we have also observed an approximately 41 kDa RNA-binding activity that shared the properties of size and nuclear restriction with hnRNP C (20). We have confirmed that hnRNP C is a constituent of this activity by immunoprecipitation with a specific monoclonal antibody. The ability of hnRNP C to bind AUUUA-containing RNAs was observed previously (4).

Using similar RNA substrates and *in vitro* crosslinking assay, two other groups have previously observed an AU-rich RNA-binding activity in nuclear extracts that is distinct from hnRNP A1 (4, 37), but did not address the possibility that this activity is also present in the cytoplasm. Another group has recently reported that a factor termed AUBF, which has a great deal of similarity to AU-A, consists of the hnRNP A1 protein (38). These investigators used a subcellular fractionation procedure that also resulted in the detection of hnRNP C in cytoplasmic samples. This observation is inconsistent with the nuclear restriction of hnRNP C that has been observed by ourselves and others (20, 39–41), and suggests that the cytoplasmic extracts used in their experiments might have contained material originating from the nucleus. We have observed by immunoblotting that little or no hnRNP A1 was present in cytoplasmic fractions, prepared as described herein, of unstimulated Jurkat cells (data not shown). However, we observed that significantly more hnRNP A1 was extracted from nuclei using the lysis buffer described by Hamilton, *et al.* (38). It would be difficult under these conditions to distinguish hnRNP A1, an abundant protein that is able to bind AUUUA repeats, from the actual constituent(s) of AUBF/AU-A.

Because our assay is amenable to study of subcellular fractions, we were able to determine that cytoplasmic AU-A is found predominantly in complexes that cosediment with 60S ribosomal subunits and 80S ribosomes. This suggests that AU-A is likely part of an mRNP that is a substrate of nucleocytoplasmic transport and/or cytoplasmic metabolism. We have not yet determined, though, whether AU-A is associated with ribosomes or supramolecular complexes that are entirely distinct. Following

treatment with RNA polymerase II inhibitors for 3 hours, we observed a significant accumulation of cytoplasmic AU-A. The simplest explanation for this observation is that following arrest of transcription the level of free nuclear AU-A (not bound to RNA) increases. This accumulation subsequently results in higher levels of cytoplasmic AU-A by reestablishment of the equilibrium ratio of free AU-A on both sides of the nuclear pore.

Our results imply that AU-A is actively exported from the nucleus. AU-A is probably actively imported from the cytoplasm, but our experiments have not proved this point. The process of nuclear import can be divided into at least two steps that have differential dependence on temperature, ATP and cytosolic factors (32, 42, 43). In cells cultured at 4°C, nuclear import substrates associate with the outer nuclear membrane, but are not transported through the nuclear pore complex (32). We expected, therefore, to observe an accumulation of AU-A at the nuclear membrane when transcriptional inhibition was released and cells were cultured at 4°C. However, when we performed this experiment, complexes containing AU-A remained intact. This suggests involvement of a signal originating from the nucleus in regulation of cytoplasmic complexes containing AU-A.

Quantitative analysis of our data argues against several trivial explanations for the changes in observed levels of cytoplasmic AU-A. We believe that our results were not due to nuclear leakage caused by experimental manipulations. Arrest of RNA polymerase II transcription led to cytoplasmic AU-A levels of $7.5 \pm 2.3\%$ ($n=4$) compared to a level of $2.8 \pm 1.3\%$ ($n=6$) in control cells. In contrast, cytoplasmic hnRNP C levels were $1.0 \pm 0.7\%$ in both groups. The nuclear restriction of hnRNP C has been observed in cells fixed for immunofluorescence and cells fractionated by methods different from those used in this work (20, 39, 40). hnRNP C is a particularly useful internal control for both nuclear leakage during extract preparation and the proportion of mitotic cells in bulk culture. The accumulation of AU-A in the cytoplasm was not related to bulk changes in either the amount of protein present in the 'polysomal' fraction ($r^2=0.33$, $n=10$), or the percentage of total cellular protein in that fraction ($r^2=0.25$, $n=10$). Treatment with inhibitors of transcription did not alter total AU-A activity levels relative to total cellular protein levels over the course of our experiments ($r^2=0.91$, $n=10$). Thus, the effect we observed was not due to differential expression or degradation of AU-A relative to other proteins.

It will be interesting to determine whether AU-A has an active role in cytoplasmic mRNA metabolism, or is only a structural component of cytoplasmic mRNPs. One possibility is that interchange of mRNA-binding proteins on AUUUA multimers may be involved in regulation of degradation or translation rate. For example, mRNA stability may be regulated by competitive binding to specific sites between AU-A and proteins that determine rapid degradation of lymphokine (15) or protooncogene mRNAs (41, 44). Increased cytoplasmic AU-A following inhibition of transcription would thereby enhance cytoplasmic mRNA stability, as observed previously for hybrid RNAs containing the AU-rich region of the *c-fos* 3' UTR (5).

ACKNOWLEDGEMENTS

We are grateful to Serafín Piñol-Roma, Doug Portman and Gideon Dreyfuss for the generous gifts of monoclonal antibodies and purified hnRNP A1 protein. We thank Paul Bohjanen, Larry Boise, Jonathan Green and Xiaohong Mao for helpful discussions,

and Bronisława Petryniak for technical assistance. D.A.K. is supported by a fellowship from the Irvington Institute. This work was supported in part by grant CA54521 from the National Cancer Institute to T.L., and by a grant from the March of Dimes Foundation to D.W.C.

42. Moore, M.S. and Blobel, G. (1992) *Cell*, **69**, 939–950.
43. Newmeyer, D.D. and Forbes, D.J. (1988) *Cell*, **52**, 641–653.
44. Brewer, G. (1991) *Mol. Cell. Biol.*, **11**, 2460–2466.

REFERENCES

1. Caput, D., Beutler, B., Hartog, K., Thayer, R., Brown-Shimer, S. and Cerami, A. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 1670–1674.
2. Shaw, G. and Kamen, R. (1986) *Cell*, **46**, 659–667.
3. Raymond, B., Atwater, J.A. and Verma, I.M. (1989) *Onc. Res.*, **5**, 1–12.
4. Vakalopoulou, E., Schaack, J. and Shenk, T. (1991) *Mol. Cell. Biol.*, **11**, 3355–3364.
5. Shyu, A.-B., Greenberg, M.E. and Belasco, J.G. (1989) *Genes Dev.*, **3**, 60–72.
6. Wilson, T. and Treisman, R. (1988) *Nature (London)*, **336**, 396–399.
7. Poppel, K., Vinci, J.M. and Baglioni, C. (1991) *J. Exp. Med.*, **173**, 349–355.
8. Brewer, G. and Ross, J. (1988) *Mol. Cell. Biol.*, **8**, 1697–1708.
9. Savant-Bhonsale, S. and Cleveland, D.W. (1992) *Genes Dev.*, **6**, 1927–1939.
10. Kruys, V., Wathelet, M., Poupart, P., Contreras, R., Fiers, W., Content, J. and Huez, G. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 6030–6034.
11. Kruys, V., Marinx, O., Shaw, G., Deschamps, J. and Huez, G. (1989) *Science*, **245**, 852–855.
12. Kruys, V.I., Wathelet, M.G. and Huez, G.A. (1988) *Gene*, **72**, 191–200.
13. Han, J., Brown, T. and Beutler, B. (1990) *J. Exp. Med.*, **171**, 465–475.
14. Han, J. and Beutler, B. (1990) *Eur. Cytokine Net.*, **1**, 71–75.
15. Bohjanen, P.R., Petryniak, B., June, C.H., Thompson, C.B. and Lindsten, T. (1991) *Mol. Cell. Biol.*, **11**, 3288–3295.
16. Bohjanen, P.R., Petryniak, B., June, C.H., Thompson, C.B. and Lindsten, T. (1992) *J. Biol. Chem.*, **267**, 6302–6309.
17. Malter, J.S. (1989) *Science*, **246**, 664–666.
18. Bickel, M., Iwai, Y., Pluznik, D.H. and Cohen, R.B. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 10001–10005.
19. Port, J.D., Huang, L.-Y. and Malbon, C.C. (1992) *J. Biol. Chem.*, **267**, 24103–24108.
20. Piñol-Roma, S. and Dreyfuss, G. (1992) *Nature (London)*, **355**, 730–732.
21. Brewer, G. and Ross, J. (1990) *Meth. Enzymol.*, **181**, 202–209.
22. Beebe, T.J.C. (1986) In A. J. MacGillivray and G. D. Birnie (ed.), *Nuclear Structures: Isolation and Characterization*. Butterworths, London, pp. 100–117.
23. Choi, Y.D. and Dreyfuss, G. (1984) *J. Cell Biol.*, **99**, 1997–2004.
24. Dreyfuss, G., Choi, Y.D. and Adam, S.A. (1984) *Mol. Cell. Biol.*, **4**, 1104–1114.
25. Schreier, M.H. and Stachelin, T. (1973) *J. Mol. Biol.*, **73**, 329–349.
26. Peltz, S.W., Brewer, G., Kobs, G. and Ross, J. (1987) *J. Biol. Chem.*, **262**, 9382–9388.
27. Ross, J., Kobs, G., Brewer, G. and Peltz, S.W. (1987) *J. Biol. Chem.*, **262**, 9374–9381.
28. Bernstein, P.L., Herrick, D.J., Prokipcak, R.D. and Ross, J. (1992) *Genes Dev.*, **6**, 642–654.
29. Pandey, N.B., Sun, J.-H. and Marzluff, W.F. (1991) *Nucl. Acids Res.*, **19**, 5653–5659.
30. Billings, P.B. and Hoch, S.O. (1984) *J. Biol. Chem.*, **259**, 12850–12856.
31. Choi, Y.D. and Dreyfuss, G. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7471–7475.
32. Richardson, W.D., Mills, A.D., Dilworth, S.M., Laskey, R.A. and Dingwall, C. (1988) *Cell*, **52**, 655–664.
33. Mirfakhrai, M. and Weiner, A.M. (1993) *Nucl. Acids Res.*, **21**, 3591–3592.
34. Mehlin, H., Daneholt, B. and Skoglund, U. (1992) *Cell*, **69**, 605–613.
35. Piñol-Roma, S. and Dreyfuss, G. (1991) *Science*, **253**, 312–314.
36. Swanson, M.S. and Dreyfuss, G. (1988) *EMBO J.*, **7**, 3519–3529.
37. Myer, V.E., Lee, S.I. and Steitz, J.A. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 1296–1300.
38. Hamilton, B.J., Nagy, E., Malter, J.S., Arrick, B.A. and Rigby, W.F.C. (1993) *J. Biol. Chem.*, **268**, 8881–8887.
39. Leser, G.P., Escara-Wilke, J. and Martin, T.E. (1984) *J. Biol. Chem.*, **259**, 1827–1833.
40. Preugschat, F. and Wold, B. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 9669–9673.
41. Zhang, W., Wagner, B.J., Ehrenman, K., Schaeffer, A.W., DeMaria, C.T., Crater, D., DeHaven, K., Long, L. and Brewer, G. (1993) *Mol. Cell. Biol.*, **13**, 7652–7665.