Modulation of AUUUA Response Element Binding by Heterogeneous Nuclear Ribonucleoprotein A1 in Human T Lymphocytes

THE ROLES OF CYTOPLASMIC LOCATION, TRANSCRIPTION, AND PHOSPHORYLATION*

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The heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) shuttles between the cytoplasm and nucleus and plays important roles in RNA metabolism. Whereas nuclear hnRNP A1 has been shown to bind intronic sequences and modulate splicing, cytoplasmic hnRNP A1 is associated with poly(A)+ RNA, indicating different RNA ligand specificity. Previous studies indicated that cytoplasmic hnRNP A1 is capable of high-affinity binding of reiterated AUUUA sequences (ARE) that have been shown to modulate mRNA turnover and translation. Through a combination of two-dimensional gel and proteolysis studies, we establish hnRNP A1 (or structurally related proteins that are post-translationally regulated in an identical manner) as the dominant cytoplasmic protein in human T lymphocytes capable of interacting with the ARE contained within the context of full-length granulocyte-macrophage colony-stimulating factor mRNA. We additionally demonstrate that cytoplasmic hnRNP A1 preferentially binds ARE relative to pre-mRNAs in both cross-linking and mobility shift experiments. RNA polymerase II inhibition increased the binding of ARE (AUBP activity) and poly(U)-Sepharose by cytoplasmic hnRNP A1, while nuclear hnRNP A1 binding was unaffected. Nuclear and cytoplasmic hnRNP A1 could be distinguished by the differential sensitivity of their RNA binding to diamide and N-ethylmaleimide. The increase in AUBP activity of cytoplasmic hnRNP A1 following RNA polymerase II inhibition correlated with serine-threonine dephosphorylation, as determined by inhibitor and metabolic labeling studies. Thus, cytoplasmic and nuclear hnRNP A1 exhibit different RNA binding profiles, perhaps transduced through serine-threonine phosphorylation. These findings are relevant to the specific ability of hnRNP A1 to serve distinct roles in post-transcriptional regulation of gene expression in both the nucleus and cytoplasm.
ing proteins could not be excluded.

Other studies have suggested the functional relevance of cytoplasmic hnRNP A1-mRNA interactions in terms of mRNA stability. RNA polymerase II inhibition decreases ARE-dependent mRNA turnover of c-fos mRNA (33), paralleling the marked increase in cytoplasmic hnRNP A1 levels (14, 18). In addition, stabilization of interleukin-2 mRNA turnover in the MLA-144 cell line is associated with a proviral insertion that enhanced hnRNP A1 binding to its ARE relative to native interleukin-2 or GM-CSF (34, 35). Increased ARE binding by hnRNP A1 thus correlates with mRNA stability in vivo, similar to observations made with AUBF, a similarly sized protein, in an *in vitro* model of ARE-dependent mRNA turnover (31).

These past studies generated several important questions regarding the cytoplasmic 35-kDa AUBP/hnRNP A1 and regulation of its RNA binding specificity in terms of understanding ARE-dependent mRNA turnover. Is the cytoplasmic 35-kDa AUBP activity made up of other proteins besides hnRNP A1 (17, 36)? If hnRNP A1 is an important cytoplasmic AUBP, do its cytoplasmic and nuclear forms differ in their RNA binding specificity? Third, what modulates the quantitative increase in cytoplasmic hnRNP A1 that accompanies RNA polymerase II inhibition and how is this effect transduced?

In this paper, we establish that hnRNP A1 (or closely related proteins that are post-translationally regulated in an identical manner) is the dominant cytoplasmic protein capable of interacting with the ARE contained within full-length GM-CSF mRNA. Moreover, AUBP activity of cytoplasmic and nuclear hnRNP A1 are differentially regulated: RNA polymerase II inhibition appears to increase the binding specificity of cytoplasmic hnRNP A1 for ARE relative to other RNA ligands to a greater degree than its nuclear counterpart. Metabolic labeling and phosphatase inhibitor studies indicate that RNA binding by cytoplasmic hnRNP A1 is regulated by serine-kinase phosphorylation. These findings are relevant to the regulation of RNA-protein interactions as well as the specific ability of hnRNP A1 to serve distinct roles in post-transcriptional regulation of gene expression in both the nucleus and cytoplasm.

### EXPERIMENTAL PROCEDURES

#### Materials—

Actinomycin D, diamide, N-ethylmaleimide, β-mercaptoethanol, and trypsin were purchased from Sigma. Poly(U)-Sepharose and cyanogen bromide-activated Sepharose beads were purchased from Pharmacia. Protease inhibitors (10 mM HEPES, pH 7.9, 150 mM KCl, 0.2 mM EDTA, 25% glycerol) were purchased from Boehringer Mannheim. RNA probes (8 × 10^6 cpm/mg) were synthesized by T7 RNA polymerase transcription of this plasmid linearized with EcoRI. The Δ2R1 probe, which contains a sequence found in the β'-untranslated region of GM-CSF mRNA, was produced by T7 RNA polymerase transcription of EcoRI-linearized pT7/73α19 vector with 4 reiterated AUUUA sequences in the BomHI site of the multiple cloning site, with the antisense transcript containing 4 UAAAU sequences called Δ2H3 (29). Δ2R1-U→C has 4 reiterated AUUUA instead of AUAUU sequences. Each was generously provided by James Malter (29).

#### a[^32]P-Labeled mRNAs with specific activity of >10^8 cpm/mg RNA were prepared by *in vitro* transcription in the presence of 50 μCi of [α-[^32]P]UTP (3000 Ci/mmol) from NEON Life Science Products, 0.0125 mM UTP, 2.5 mM ATP, GTP, and CTP from Boehringer Mannheim. RNA probes (8 × 10^6 cpm) were incubated with 10–20 μg of cytoplasmic extract or 2–5 μg of nucleoprotein extract in 12 mM Hepes, pH 7.9, 15 mM KCl, 0.2 μM dithiothreitol, 0.2 μg/ml yeast tRNA, and 10% glycerol and 10 μl of RNA polymerase II (Boehringer Mannheim) for 30 min at 30 °C. DNA cross-linking was performed at 4 °C using a Stratagene UV Stratalinker 1800 (5 min, 3000 microwatts/cm²) followed by RNase digestion (10 units of RNase T1 and 20 μg of RNase A) for 30 min at 37 °C (18). The sample was analyzed under denaturing conditions by 12% SDS-PAGE and either dried on a gel dryer or transferred to nitrocellulose (Schleicher & Schuell, 0.4 μm) in 10 mM CAPS, pH 11.0, 15% methanol using a Bio-Rad transblot apparatus at 20 V × 1.5 h followed by autoradiography. Electrophoretic mobility shift assays were performed as described previously (30).

Analysis of AUBU-binding Proteins by Partial Proteolysis—Cytoplasmic extracts prepared from 20-h PHA (1 μg/ml) or 2-h actinomycin D (5 μg/ml) were incubated with [α-[^32]P]UTP (3000 Ci/mmol) from NEON Life Science Products, 0.0125 mM UTP, 2.5 mM ATP, GTP, and CTP (Boehringer Mannheim) and 1 μg/ml trypsin (0.1 mg/ml) or RNAse A at 37 °C for 3 h at room temperature. Trypsin digestion was stopped by the addition of SDS-PAGE sample buffer and followed by analysis under denaturing conditions by 12% SDS-PAGE.

Poly(U)-Sepharose Binding Assay—Assays were performed as described previously (5). Cytoplasmic (100 μg) or nucleoplasmic (25 μg) extracts prepared from 20-h PHA + 2-h actinomycin D (5 μg/ml) or ethanol control were incubated with poly(U)-Sepharose beads in 12 mM Hepes pH 7.9, 0.2 μM dithiothreitol, 0.2 μg/ml yeast tRNA, and the specified concentration of NaCl at 15 min at room temperature with gentle agitation. The quantity of poly(U)-Sepharose beads (50 μl packed) used was in excess of the amount capable of completely depleting all hnRNP A1 from lysates when incubated in the absence of salt. Beads were washed extensively with the same binding buffer (6 × 500 μl) before addition of SDS-PAGE sample buffer and boiling. Samples were analyzed by 12% SDS-PAGE denaturing gel. Proteins were electrophoresed as described above and immunoblotted with 4B10 to detect hnRNP A1. Similar results were obtained with cytoplasmic lysates whose total hnRNP A1 levels were adjusted to have equivalent levels of hnRNP A1 between control and actinomycin D-treated samples.

### 2-D NEPHGE/SDS-PAGE—

Two-dimensional NEPHGE was done as described by O’Farrell et al. (40). 100 μg of cytoplasmic extracts were separated in the first dimension with pH 3–10 ampholines (Bio-Rad) at 400 V for 135 min (900 volt-h). The second dimension was reducing denaturing 12% SDS-PAGE. Proteins were electrophoresed as described above followed by autoradiography or immunoblotting.
The AUUUA sequences in GM-CSF and Δ2R1 are underlined. Δ2R1.U → C has reiterated AUCUA instead of AUUUA sequences. The β-globin probe contains the first intron and exon of human β-globin as well as the first 23 nucleotides of the second exon, shown as (N)23. The polypyrimidine tract at the 3′ end of the first intron of β-globin is underlined. The DUP33Y5 transcript contains 2 repeats of the first intron of β-globin, of which the latter is identical to that found in the first intron of β-globin pre-mRNA. The first intronic polypyrimidine tract (bracketed) of DUP33Y5 is mutated at sites (bold type, underlined) to contain oligouridine stretches of 5 and 6 bases each. These two intronic sequences are separated by 33 nucleotides (denoted as (N)33) which contains sequences of the first and second exon of β-globin (19). Thus, DUP33Y5 is very similar to the β-globin, with two intronic sequences that are identical to that found in the β-globin probe, but for the presence of 28 untranslated pyrimidines in the first.

| Sequence   | RNA sequences used in AUBP assays |
|------------|----------------------------------|
| Δ2R1       | 5′-GAGAACCUCUAUAAUUAAUAUAGCUGUGG-3′ |
| Δ2R1.U → C | 5′-GGACUCUAUAAUAUAAUAUAAUAUAGCUGUGG-3′ |
| Δ2R3       | 5′-CCCGCUAAUAAUAAUAUAAUAUAGCUGUGG-3′ |
| GM-CSF     | 5′-AUG-(N)23-AAGAAGGGAAAUUUUUAUCUG |
| β-Globin   | 5′-(N)24 UUGGUUCAAGGUUAAAGCAGAGG |
| DUP33Y5    | 5′-(N)24 UUGGUUCAAGGUUAAAGCAGAGG |

**Preparation of Polyclonal Antibody against HnRNP A1 C-Terminus**—Polyclonal antibody to the C-terminal 18 amino acids of hnRNP A1 (ACT-1) was raised in a rabbit through immunization with KLH-C-terminal peptide complex, where disulfide linkage of the peptide had been used to immobilize the peptide. Eluted antibody was specific for hnRNP A1 and did not react with any other hnRNP proteins. Affinity-purified antibody was covalently cross-linked to cytochrome c-activated Sepharose beads by manufacturer’s instructions (Pharmacia).

**In Vivo Labeling and Immunoprecipitation of HnRNP A1—**Human peripheral blood lymphocytes (1 × 10⁶) were cultured as described above for 20 h before washing four times with phosphate-free medium. Cells were cultured 1 h in phosphate-free RPMI (Bio-Labs, Rockville, MD) plus 10% dialyzed fetal calf serum before addition of 10 mCi of [α-²⁵³P]PO₄ for 3.5 h. Actinomycin D (5 µg/mL) or ethanol control was added for 2 h before preparing cytoplasmic extracts. Extracts were precleared with protein A-Sepharose beads before immunoprecipitation with ACT-1-Sepharose beads for 4 h at 4°C on rotator in 10 ml Tris-HCl, pH 7.4, 100 mM NaCl, 2.5 mM MgCl₂, 0.5% Triton X-100, 1 mM Pefabloc, and 1 µg/mL each of leupeptin and pepstatin A. The beads were washed extensively before addition of SDS-PAGE sample buffer and boiling. Samples were electrophoresed by 12% SDS-PAGE and electrotransferred to nitrocellulose before autoradiography and immunoblotting. Under these conditions, lysates are cleared of all immunoreactive hnRNP A1, as measured by Western blotting of immunodepleted lysates with 4B10 or ACT-1.

**RESULTS**

**Comparison of AUBP and Pre-mRNA Binding Activity of Cytoplasmic HnRNP A1—**The relative RNA binding specificity of cytoplasmic hnRNP A1 from mitogen-activated human T lymphocytes for ARE and 3′-intron splice sites was initially analyzed by incubation with radiolabeled [³²P]UTP-RNA (full-length GM-CSF mRNA and β-globin pre-mRNA shown in Table I) followed by UV cross-linking, RNase digestion, SDS-PAGE, and electroblotting on nitrocellulose (Fig. 1), as previously described (18). Cytoplasmic lysates demonstrated qualitative differences in their RNA binding profile relative to previous studies with oligoribonucleotide probes containing ARE (18). Notably, a 35-kDa protein appeared to be the major protein labeled by UV cross-linking following incubation with pre-labeled GM-CSF, which colocalized with hnRNP A1 by subsequent immunoblotting. Second, this 35-kDa complex demonstrated a much higher degree of labeling (reflecting binding and subsequent UV cross-linking) with GM-CSF relative to that seen with a β-globin pre-mRNA transcript.

Following actinomycin D treatment, increased 35-kDa GM-CSF and β-globin binding activity was observed, but the relative binding intensity of these two RNA probes was maintained. Relative to the other proteins (68, 50, and 40 kDa) bound and labeled by these RNA, the cytoplasmic 35-kDa protein demonstrated not only the greatest level of binding, but also the greatest differential of labeling between the GM-CSF and β-globin pre-mRNA transcripts. In other experiments, the cytoplasmic 35-kDa AUBP consistently bound more effectively to β-globin than DUP33Y5 pre-mRNA (data not shown). The DUP33Y5 pre-mRNA transcript contains two 3′-splice sites, the distal one identical to that contained in the β-globin pre-mRNA, while its proximal site contains several oligouridine sequences (Table I), thereby controlling for oligouridine-dependent effects on RNA binding and cross-linking. From these studies, we infer that the increased binding of the 35-kDa AUBP to the GM-CSF mRNA sequence relative to β-globin pre-mRNA cannot be accounted for by the presence of oligouridine sequences that enhance binding or cross-linking efficiency.

**Characterization of Cytoplasmic 35-kDa AUBP by 2-D NPHGE and Tryptic Mapping—**Previous immunoprecipitation studies established that hnRNP A1 is a component of the cytoplasmic 35-kDa AUBP capable of binding an oligoribonucleotide probe, η2R1 (Table I), which contains four of the five AUUUA consecutive repeats found in GM-CSF (29). The demonstration that 35-kDa cytoplasmic proteins appear to interact with GM-CSF RNA to a greater degree than β-globin pre-mRNA do not identify if this differential binding pattern involves the same 35-kDa proteins, including hnRNP A1. To resolve this issue, as well as examine the ARE specificity of the interaction of the 35-kDa proteins with GM-CSF RNA, we performed two-dimensional gel electrophoresis (NPHGE) of cytoplasmic lysates following UV cross-linking to radiolabeled probes.
lymphocytes were resolved by 2-D NEPHGE/SDS-PAGE following UV cross-linking to [32P]-labeled GM-CSF, an identical pattern was observed with the probe were identified, ranging in pI from 6.5 to 8.3 (Fig. 2). An specificity, and identical tryptic mapping following UV cross-linking of the labeled 35-kDa AUBP (pI 6–8.3) were resolved by UV cross-linking of 35-kDa AUBP (pI 6–8.3) to GM-CSF RNA, and Δ2R1 RNA probes, and analyzed by autoradiography.

Four major 35-kDa proteins capable of binding the GM-CSF probe were identified, ranging in pl from 6.5 to 8.3 (Fig. 2). An identical pattern was observed with the β-globin RNA, albeit with diminished intensity for each RNA-protein complex. These data indicate that β-globin and GM-CSF RNA interact with the same 35-kDa proteins, although each protein preferentially bound GM-CSF. In contrast, the binding of β-globin and GM-CSF RNA to the 68-kDa protein was equivalent, while β-globin RNA selectively interacted with a 50-kDa protein (pl ~ 6.5) to a greater degree than either of the ARE-containing probes. The enhanced binding of GM-CSF RNA by the 35-kDa AUBP relative to β-globin RNA was similarly seen in electrophoretic mobility shift experiments, indicating these differences were independent of UV cross-linking. Moreover, this preferential binding of GM-CSF and degree of retardation of the 35 kDa was identical to that seen with recombinant hnRNP A1 (Fig. 3A).

The ARE specificity of the binding of the 35-kDa AUBP for GM-CSF was further suggested by binding experiments with radiolabeled Δ2R1 RNA, which demonstrated a nearly identical pattern and intensity of 35-kDa binding proteins to GM-CSF RNA except for the presence of a fifth, more acidic isoform. Both recombinant hnRNP A1 and the 35-kDa AUBP demonstrated identical fine ARE specificity as little or no binding is seen with Δ2R1:U→C RNA, which contains 4 reiterated AU-CUA sequences (Fig. 3B). The ARE-specific binding of GM-CSF RNA by hnRNP A1 was further established by the superior ability of unlabeled Δ2R1, but not its antisense Δ2H3 (Table I), to compete for GM-CSF RNA binding by recombinant hnRNP A1 (Fig. 3C). Moreover, this pattern of competition was identical to that observed for the 35-kDa AUBP. Finally, tryptic digestion of the 35-kDa AUBP following UV cross-linking to GM-CSF RNA yielded a trypsin-resistant 28-kDa AUBP (Fig. 3D), identical to that seen with recombinant hnRNP A1 (18).

The four isoforms of the cytoplasmic 35-kDa AUBP capable of interacting with the ARE found in GM-CSF mRNA thus demonstrate equivalent fine binding specificity to that seen with hnRNP A1, and are identical to hnRNP A1 in terms of their size, RNA binding specificity, the size of their RNA-binding sites, as well as their sites of trypsin sensitivity when bound to RNA. We also conclude that, in the context of full-length GM-CSF RNA, cytoplasmic 35-kDa protein and hnRNP A1 bind this ARE to a greater degree than the splice sites found in β-globin pre-mRNA.

Colocalization of HnRNP A1 with 35-kDa AUBP Activity by 2-D NEPHGE/Immunoblotting—To further identify the components of the 35-kDa AUBP activity as hnRNP A1, two-dimensional NEPHGE of an AUBP reaction followed by Western blotting was performed (Fig. 4, left panels). Five cytoplasmic 35-kDa AUBP (pl 6–8.3) were resolved by UV cross-linking of the labeled Δ2R1 probe added to PHA-activated cytoplasmic lysates. As we had shown nearly identical AUBP activity, ARE specificity, and identical tryptic mapping following UV cross-linking to GM-CSF and Δ2R1 probes (18), the latter was chosen to minimize any potential steric effect of cross-linked RNA retained despite extensive RNase digestion. The most basic
35-kDa AUBP isoform co-localized with the most acidic hnRNP A1 isoform (arrow) by subsequent immunoblotting. Following actinomycin D treatment, cytoplasmic hnRNP A1 levels and 35-kDa AUBP activity increased, with the appearance of two additional 35-kDa AUBP isoforms (arrows) that co-localized with hnRNP A1 by immunoblotting (Fig. 4, right panels).

These data indicate that AUBP activity of hnRNP A1 either resides in its more acidic isoforms or that altered migration is secondary to the formation of RNA-hnRNP A1 complexes. Not all cytoplasmic hnRNP A1 can be cross-linked to ARE under these conditions, indicating that cytoplasmic hnRNP A1 is heterogeneous in its ability to bind reiterated ARE. Thus, the failure to colocalize more acidic 35-kDa AUBP isoforms with hnRNP A1 may reflect the orders of magnitude greater sensitivity afforded by 32P relative to immunoblotting. In addition, UV-mediated label transfer of RNA to hnRNP A1 may partially mask the epitope recognized by the 4B10 antibody, as described previously (18). Nevertheless, the presence of other 35-kDa AUBPs besides hnRNP A-type proteins cannot be definitively excluded, although the proteolysis and binding specificity studies (Fig. 3) would indicate that hnRNP A1 or a closely related protein represents most, if not all, of the 35-kDa AUBP activity detected with the GM-CSF RNA probe (9, 18). In that regard, we have excluded hnRNP A2 as a GM-CSF RNA-binding protein, as immunoblotting failed to demonstrate any colocalization with AUBP activity.2

Modulation of RNA Binding Affinity of 35-kDa AUBP and HnRNP A1 by Actinomycin D Treatment—These data indicate: (i) cytoplasmic 35-kDa AUBP activity consists of hnRNP A1 alone or along with very closely related proteins; (ii) each of the 35-kDa proteins with AUBP activity preferentially binds the ARE of GM-CSF or Δ2R1 relative to β-globin pre-mRNA. To confirm the latter with both cytoplasmic and nuclear hnRNP A1, as well as to determine if actinomycin D induces selective differences in hnRNP A1 binding, we examined the ability of pre-mRNA (β-globin, DUP33Y5) probes to compete for ARE binding (Fig. 5). As stated earlier, the DUP33Y5 pre-mRNA probe was chosen because it has two 3′-splice sites, the distal one identical to that contained in the β-globin pre-mRNA, while its proximal site contains several oligouridine sequences (Table 1), thereby providing a control for oligouridine binding and cross-linking between these three RNA probes. Autoradiography followed by immunoblotting permitted colocalization of hnRNP A1 with the 35-kDa AUBP.

In the nuclear fraction, equivalent levels of 40- and 35-kDa AUBP were observed, with equivalent levels of each AUBP in the nuleoplasm, in contrast to that seen with cytoplasmic fractions. In each, the binding of radiolabeled GM-CSF to the 35-kDa AUBP was competed less well by β-globin and DUP33Y5 RNA than by GM-CSF itself (Fig. 5, A and B). Unlabeled β-globin RNA competed for GM-CSF binding better than DUP33Y5, suggesting that the enhanced binding of hnRNP A1 to GM-CSF RNA relative to these pre-mRNAs was not dependent on the presence of oligouridine sequences. Following actinomycin D treatment, the competition profiles of cytoplasmic and nuclear 35-kDa AUBP activity/hnRNP A1 were differentially modulated: cytoplasmic 35-kDa binding to radiolabeled GM-CSF RNA was less well competed by unlabeled β-globin and DUP33Y5 RNA, while competition by GM-CSF was relatively unaffected (Fig. 5A). In contrast, actinomycin D treatment was associated with little or no alteration of nuclear 35-kDa AUBP or its competition by β-globin and DUP33Y5 RNA in 10 experiments and variable effects on nuclear 40-kDa AUBP activity (Fig. 5B). These data suggest that actinomycin D treatment selectively enhanced cytoplasmic 35-kDa AUBP activity, not only by increasing the level of hnRNP A1 (or related proteins) in the cytoplasm, but also by increasing its ability to interact with ARE.

The interpretation that actinomycin D treatment (and hence RNA polymerase II inhibition) qualitatively altered cytoplas-
modic 35-kDa AUBP/hnRNP A1 binding to RNA was supported by finding that actinomycin D treatment increased the salt concentration at which cytoplasmic 35-kDa binding to radiolabeled GM-CSF RNA could be measured (Fig. 5C). In the absence of actinomycin D treatment, cytoplasmic 35-kDa AUBP binding activity markedly decreased when the incubation was carried out in the presence of 100 mM KCl. After actinomycin D treatment, a high level of cytoplasmic binding was maintained with incubations at 200 mM KCl. Similar effects were observed with DRB, which inhibits RNA polymerase II in a manner distinct from actinomycin D (41). Moreover, treatment with actinomycin D at concentrations which inhibit RNA polymerase I or drugs that inhibit translation, did not induce a similar effect (data not shown). Thus, treatment with drugs at concentrations that inhibit RNA polymerase II qualitatively alter cytoplasmic hnRNP A1 binding to ARE. This same profile of drug action was used to define the conditions that increased cytoplasmic hnRNP A1 in HeLa cells (42). Thus, qualitative (RNA binding) changes in hnRNP A1 accompany its cytoplasmic increase following transcriptional inhibition, suggesting their potential linkage.

Alterations in Cytoplasmic hnRNP A1 Binding to Poly(U)-Sepharose by Actinomycin D—These experiments demonstrate that actinomycin D treatment modulates the ability of cytoplasmic 35-kDa AUBP, which appears to consist primarily of hnRNP A1 or a closely related protein, to bind to radiolabeled GM-CSF in a UV cross-linking assay. These data do not unambiguously establish that the increase in GM-CSF RNA binding induced by RNA polymerase II inhibition is only by hnRNP A1 or determine its dependence on an UV cross-linking assay. Additionally, these data do not easily permit direct controlling for the change in cytoplasmic levels of hnRNP A1 induced by actinomycin D treatment. These issues were addressed as described previously (5), by examining the salt dependence of binding of cytoplasmic and nuclear hnRNP A1 to immobilized ribohomopolymer (poly(U)-Sepharose) (Fig. 6A).

Cytoplasmic hnRNP A1 binding (from PHA-activated lymphocytes) to poly(U)-Sepharose was not detected when incubation and wash buffers contained NaCl concentrations above 0.1 M. In contrast, measurable nuclear binding was detectable with salt concentrations up to 0.5 M. Actinomycin D treatment increased cytoplasmic hnRNP A1 binding, with a pattern of salt-sensitive binding equivalent to that found in the nuclear fraction. These effects are mediated by increased binding affinity rather than increased levels of cytoplasmic hnRNP A1, as they are still evident when poly(U) binding of hnRNP A1 is plotted as a percentage of the total hnRNP A1 present within each fraction (Fig. 6B). Therefore, nuclear and cytoplasmic hnRNP A1 demonstrate different affinities for poly(U)-Sepharose, with RNA polymerase II inhibition selectively increasing the poly(U) binding affinity of cytoplasmic hnRNP A1. Thus, RNA polymerase II inhibition induces qualitative differences in the RNA binding of cytoplasmic hnRNP A1 as measured with both cross-linking as well as non-cross-linking approaches. This coregulation of RNA binding of cytoplasmic 35-kDa AUBP/hnRNP A1 provides further evidence that these proteins are likely to be the same or highly related proteins.

Differential Sensitivity of Cytoplasmic and Nuclear HnRNP A1 RNA Binding Activity to Oxidation—In the absence of RNA polymerase II inhibition, the binding of nuclear hnRNP A1 to poly(U)-Sepharose was markedly less sensitive to ionic interactions than its cytoplasmic counterpart. Although our studies indicated that nuclear and cytoplasmic hnRNP A1 differ considerably in their 35-kDa AUBP activity following actinomycin D treatment (Fig. 5), the poly(U) binding data are also consistent with the interpretation that RNA polymerase II inhibition merely increases the nuclear export rate and/or cytoplasmic retention of recently exported nuclear hnRNP A1, rather than alters its RNA binding activity.

Alternatively, cytoplasmic and nuclear hnRNP A1 may differ in some other fundamental fashion that accounts for their apparent differences in ARE binding activity. This interpretation is supported by finding differential sensitivity of cytoplasmic and nuclear 35-kDa/hnRNP A1 AUBP activity to oxidation (Fig. 7A). Following actinomycin D treatment, cytoplasmic 35-kDa/hnRNP A1 AUBP activity was quite sensitive to treatment with diamide (completely lost after incubation with 5 mM diamide), while nuclear 35-kDa AUBP activity was relatively insensitive, requiring a 4-fold higher concentration to exert an equivalent effect. The specificity of this effect for oxidation was confirmed by its prevention by concurrent treatment with β-mercaptoethanol. Similarly, diamide (5 mM) treatment com-
completely eliminated cytoplasmic hnRNP A1 binding to poly(U)-Sepharose, while nuclear hnRNP A1 was unaffected (Fig. 7B).

Similar effects were found with N-ethylmaleimide, and maintained when nuclear and cytoplasmic extraction buffers contain equivalent amounts of dithiothreitol or in the presence of equivalent amounts of cytoplasmic and nuclear lysates from hnRNP A1-deficient cell lines (43) to exclude nonspecific effects of different subcellular fractions (data not shown).

These studies support the interpretation that reduced sulfhydryl groups are necessary for ARE and poly(U) binding by hnRNP A1. This differential sensitivity of RNA binding by cytoplasmic and nuclear hnRNP A1 suggests that nuclear export of hnRNP A1 to the cytoplasm is associated with a change in the accessibility of thiols to oxidation or alkylation, perhaps reflecting a change in structure. This interpretation is consistent with the considerable conformational change and elongation of mRNP complexes that contain hnRNP A1-type proteins (hrp 36) as they translocate through the nuclear pore to the cytoplasm (44, 45). Alternatively, differences in oxidative sensitivity of nuclear and cytoplasmic forms of hnRNP A1 may be mediated through post-translational modification and/or protein-protein interactions that include the increased presence of inter- or intramolecular disulfide bonds in nuclear hnRNP A1.

Whatever the mechanism, cross-linking and non-cross-linking studies define at least three RNA binding phenotypes of hnRNP A1: (i) low affinity poly(U) binding, AUBP activity, sensitive to oxidation (cytoplasmic hnRNP A1); (ii) high affinity poly(U) binding, AUBP activity, sensitive to oxidation (cytoplasmic hnRNP A1 following RNA polymerase II inhibition); (iii) high affinity poly(U) binding, AUBP activity, insensitive to oxidation (nuclear hnRNP A1). Finally, the oxidative sensitivity of cytoplasmic hnRNP A1 is unaffected by RNA polymerase II inhibition, so it does not appear to mediate the increased phosphorylation of specific serine/threonine residues play a role in regulating hnRNP A1-RNA ligand interactions. The identity of the 36- and 41-kDa phosphoproteins that appear similarly affected by actinomycin D is unknown. Given the generalized effect of RNA polymerase II inhibition on proteins that shuttle between the nucleus and cytoplasm (14, 17, 18, 36, 46), this latter finding suggests that serine/threonine dephosphorylation may be a common regulatory mechanism by which cytoplasmic retention of these proteins is mediated.

Modulation of Cytoplasmic 35-kDa/HnRNP A1 AUBP Activity by Okadaic Acid Treatment—The linkage between cytoplasmic localization, AUBP activity, and dephosphorylation of hnRNP A1 was extended utilizing the specific inhibitor of serine/threonine protein phosphatases, okadaic acid (47, 48). Cytoplasmic lysates were analyzed for both AUBP activity and levels of hnRNP A1 as a function of actinomycin D treatment (Fig. 9). Cytoplasmic 35-kDa AUBP activity was unchanged by okadaic acid treatment in the absence of actinomycin D and decreased in the presence of actinomycin D, despite increases in cytoplasmic hnRNP A1 levels. Thus, the decreased 35-kDa AUBP binding activity that accompanied okadaic acid treatment was not due to a decline in cytoplasmic levels of hnRNP A1. Under these conditions, okadaic acid treatment enhanced phosphorylation of cytoplasmic hnRNP A1 5–10-fold (data not shown), similar to previous reports (49). In contrast, nuclear 35-kDa AUBP activity was minimally affected by okadaic acid when normalized for the levels of hnRNP A1 measured by concurrent immunoblotting.

Poly(U)-Sepharose binding of hnRNP A1 was analyzed as described in the legend to Fig. 5.

**Fig. 7.** Differential sensitivity of cytoplasmic and nuclear 35-kDa AUBP activity/poly(U) binding by hnRNP A1 to oxidation. Panel A, cytoplasm (cyto) (20 μg) and nucleoplasm (nuc) (5 μg) from PHA-activated (20 h + 2-h actinomycin D) lymphocytes were sequentially incubated 10 min at 30 °C with the specified concentrations of diamide and 1% β-mercaptoethanol (β-ME), then assayed for AUBP activity with 32P-labeled GM-CSF RNA by SDS-PAGE and autoradiography. Panel B, following diamide (5 mM) treatment of cytoplasmic (100 μg) and nucleoplasm (25 μg) as described above, salt-sensitive poly(U)-Sepharose binding of hnRNP A1 was analyzed as described in the legend to Fig. 5.
Modulation of HnRNP A1 Binding to RNA

Identification of the Cytoplasmic 35-kDa AUBP as hnRNP A1 or hnRNP A-Type Proteins—Extending previous reports (18, 35), immunoblotting of one- and two-dimensional gels co-localized a significant portion of the 35-kDa AUBP activity with hnRNP A1, although other RNA-protein complexes with similar charge and mass were observed. Although definitive identification of these additional bands as hnRNP A1 was not permitted, trypsin treatment following UV cross-linking converted the entire 35-kDa AUBP activity into a 28-kDa AUBP-RNA complex that corresponds in size to the N-terminal domain/UP-1 fragment generated by a similar digestion of recombinant hnRNP A1 (18). Moreover, cytoplasmic 35-kDa AUBP activity and poly(U)-Sepharose binding by cytoplasmic hnRNP A1 demonstrated coordinate modulation by RNA polymerase II inhibition and okadaic acid treatment in vivo, as well as diamide oxidation and N-ethylmaleimide alkylation in vitro. Thus, if other proteins beside hnRNP A1 make up the cytoplasmic 35-kDa AUBP activity in normal human T lymphocytes, we conclude they are similar in size, pl, size of their ARE-binding domains, trypsin sensitivity, RNA binding specificity, sensitivity to oxidation, and post-translational regulation by serine/threonine phosphorylation (5, 7, 8, 30).

These latter studies were facilitated by the finding that the ARE in full-length GM-CSF preferentially binds and cross-links to the 35-kDa AUBP, in contrast to a truncated RNA probe (Δ2R1) containing a nearly identical ARE, which interacts equivalently with both the 35- and a 40-kDa AUBP. The potential importance of the secondary structure in enhancing AUBP-ARE interactions and mRNA turnover has been previously reported (35). In this instance, it appears that secondary structure of the RNA selectively reduced the ability of the cytosol.
cytoplasmic 40-kDa AUBP to interact with the ARE within GM-CSF, perhaps by limiting either its accessibility or the conformational changes of the RNA ligand that are necessary for 40-kDa complex formation (50, 51). Thus, while the minimal AU-rich sequence necessary for conferring instability to a defined mRNA has been characterized (52, 53), these data suggest that the context of the ARE sequence may be an important component of its function in mRNA turnover and translation.

Different RNA Binding Phenotypes of Cytoplasmic and Nuclear HnRNP A1 Defined by ARE and Poly(U) Binding—For the first time, we demonstrate that nuclear and cytoplasmic hnRNP A1 can be differentially characterized on the basis of their binding to poly(U), as well as the modulation of their AUBP activity by RNA polymerase II inhibition. These different binding profiles could be further distinguished by their differential sensitivity to oxidation by diamide, thereby defining three distinct RNA binding phenotypes: (i) nuclear (high poly(U) affinity, AUBP activity insensitive to oxidation); (ii) cytoplasmic (low poly(U) affinity, AUBP activity sensitive to oxidation); (iii) cytoplasmic following RNA polymerase II inhibition (high poly(U) affinity, increased AUBP activity sensitive to oxidation). Thus, RNA binding activity of hnRNP A1 is modulated by its cellular location as well as in response to specific stimuli.

This differential regulation of RNA binding specificity of cytoplasmic and nuclear hnRNP A1 may be mediated through post-translational modification(s) as actinomycin D-associated increases in AUBP and poly(U) binding activity of cytoplasmic hnRNP A1 correlated with its dephosphorylation on either serines or threonines. These findings suggest that serine-threonine dephosphorylation(s) plays an important role in the regulation of RNA ligand binding by hnRNP A1, but do not exclude the possible role of other factors (protein-protein interactions) in regulating RNA ligand specificity (8). In this regard, the decreased sensitivity of RNA binding by nuclear hnRNP A1 to oxidation or alkylation by N-ethylmaleimide could be accounted for by the presence of protein-protein interactions that either limit access of cysteines sterically or through the presence of intra- or intermolecular disulfide bonds. This interpretation is consistent with the observation that while nuclear hnRNP A1 exists in 40 S complexes with other hnRNP, only the hnRNP A1-type proteins (hrp 36) transit the nuclear pore to the cytoplasm (45).

Thus, differences in nuclear and cytoplasmic hnRNP A1 structure may be related to its role in nuclear export, which requires its association with mRNA (54, 55). In the larval salivary glands of Chironomus tentans, translocation of Balbiani ring mRNPs particles through the nuclear pore are associated with their extensive conformational changes and elongation (44), perhaps necessitating a reduction in the annealing activity of hrp 36, an hnRNP A1-type protein (56, 57). Decreased annealing activity of hnRNP A1 accompanies phosphorylation by protein kinase Cζ (58), which is associated with the nuclear envelope (59). Similar modulation of the annealing activity of hnRNP A1 by phosphorylation of serine 199 by protein kinase A or casein kinase have also been shown to be accompanied by substantial changes in hnRNP A1 structure (49). Thus, alteration of hnRNP A1 structure or its change in protein-protein associations, either as it translocates through the nuclear pore complex or its subsequent cytoplasmic dephosphorylation, could potentially account for the increased diamide sensitivity of cytoplasmic hnRNP A1, as well as its different RNA binding profile.

Relationship of Altered Nucleocytoplasmic Shuttling of HnRNP A1 to Changes in RNA Binding Activity—Nuclear export of hnRNP A1 requires its N-terminal RNA-binding domain while nuclear re-import is mediated by a region (amino acids 260–305) in its glycine-rich C terminus (54, 55). RNA polymerase II inhibition induces cytoplasmic accumulation of hnRNP A1, in association with poly(A)+ RNA, through decreased nuclear import (14, 42). Our studies suggest the possibility that this change in nuclear reimport may occur as a consequence of increased poly(A)+ RNA binding. In this regard, we have observed that actinomycin D treatment increases the level of cytoplasmic hnRNP A1 that can be UV cross-linked to poly(A)+ RNA in vivo in PHA-activated lymphocytes. Thus, the increased RNA binding (for ARE) of cytoplasmic hnRNP A1 induced by actinomycin D treatment may contribute to the observed rise in cytoplasmic levels: whereas nuclear export of hnRNP A1 is largely dependent on its association with poly(A)+ RNA (54), dissociation from poly(A)+ RNA may be necessary for nuclear import.

This hypothesis is suggested by the finding that the accessibility of the C terminus of hnRNP A1 is altered by ARE binding. Following UV cross-linking, hnRNP A1 cannot be efficiently immunoprecipitated (18), using either monoclonal or polyclonal antibodies that react with epitopes in the C-terminal domain of hnRNP A1. This apparent masking of the C terminus suggests its role in ARE binding, which is unsurprising as it contributes potential RNA binding sites (RGG boxes), as well as free energy and cooperativity of binding of hnRNP A1 to RNA (60–62). Given the proximity of the sequence of hnRNP A1 (amino acids 260–305) to epitopes blocked by RNA-protein interactions (54, 55), binding to poly(A)+ RNA in vivo might similarly mask this sequence from interacting with proteins that facilitate its nuclear reimport. Thus, the quantitative increase in cytoplasmic hnRNP A1 that accompanies RNA polymerase II inhibition may reflect its increased affinity for specific cytoplasmic RNA ligands.

Modulation of ARE-dependent mRNA Turnover Induced by RNA Polymerase II Inhibition—Previous work has indicated that RNA polymerase II inhibition is associated with changes in the rate of ARE-dependent mRNA turnover (33). The demonstration that RNA polymerase II inhibition is associated with a qualitative increase in the ability of cytoplasmic hnRNP A1 to bind to ARE, provides a potential mechanism to link these observations, particularly with studies that correlate hnRNP A1 binding to ARE with increased mRNA stability (35). The suggestion that hnRNP A1-ARE interactions induce considerable conformational change in its C terminus adds another potential level of complexity to ARE-dependent mRNA turnover and translation. The N- and C-terminal domains of hnRNP A1 have quite distinct effects (helicase and annealing activity, respectively) on RNA secondary structure, although intact hnRNP A1 exhibits predominantly annealing activity (56, 62–64). Post-translational modifications of the hinge region that links these two domains are associated with changes in structure and annealing activity of hnRNP A1 (49, 58, 65, 66).

Thus the functional consequence of hnRNP A1 binding to ARE may not only be regulated by the affinity of this interaction, but also the effect of hnRNP A1 on RNA structure, as governed by the relative position of the N- and C-terminal domains. Such a mechanism might account for the inability to correlate changes in AUBP activity with stimuli ( interleukin-12, CD28 (67)) modulate ARE-dependent mRNA turnover in T lymphocytes (67, 68). The delineation of the sites of dephosphorylation that increase cytoplasmic hnRNP A1 RNA binding to ARE as well as modulate its effects on RNA structure will thus generate insights into the mechanisms which permit hnRNP A1 to perform multiple roles in RNA metabolism.
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