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A General RNA-Binding Protein Complex That Includes the Cytoskeleton-associated Protein MAP 1A

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Association of mRNA with the cytoskeleton represents a fundamental aspect of RNA physiology likely involved in mRNA transport, anchoring, translation, and turnover. We report the initial characterization of a protein complex that binds RNA in a sequence-independent but size-dependent manner in vitro. The complex includes a ~160-kDa protein that is bound directly to mRNA and that appears to be either identical or highly related to a ~1600-kDa protein that binds directly to mRNA in vivo. In addition, the microtubule-associated protein, MAP 1A, a cytoskeletal associated protein is a component of this complex. We suggest that the general attachment of mRNA to the cytoskeleton may be mediated, in part, through the formation of this ribonucleoprotein complex.

INTRODUCTION

The cytoskeletal network plays important roles in the maintenance of cell shape and the transport and anchoring of cellular components. A less appreciated role of the cytoskeleton is its function as a physical anchor and transport substrate for the key mediators of gene expression in the cytoplasm—the mRNA molecules. Evidence in several experimental systems has shown this interaction to be critical for the spatial and temporal regulation of protein synthesis.

The first direct demonstration that cellular mRNAs are not free to diffuse in the cytoplasm but, rather, are attached to the cytoskeleton, was provided by experiments carried out by Penman and co-workers. They observed that actively translating polyribosomes are associated with the cellular cytoskeleton (Lenk *et al.*, 1977; Cervera *et al.*, 1981; Ornelles *et al.*, 1986). Subsequent studies confirmed these initial findings and demonstrated that isolated cytoskeletons from a variety of different cell types retained translational components including polyribosomes, translation factors, and mRNA (see Pachter [1992] for a review). Indeed, for the vast majority of mRNA species analyzed to date, 75–95% of the population has been found to be associated with the cytoskeleton (Pachter, 1992).

In addition to mRNA on polyribosomes, RNAs that are not associated with ribosomes, such as untranslated messenger ribonucleoprotein particles (mRNPs), have also been shown to be attached to the cytoskeleton in some experimental systems (Bird and Sells, 1986). Furthermore, when mRNAs are dissociated from ribosomes, the RNAs still remain associated with the cytoskeleton, indicating a direct attachment that is independent of ribosomes (Lenk *et al.*, 1977; Howe and Hershey, 1984). When exogenous RNAs are introduced into the cell, for instance after viral infections, they too have been found to associate with the cytoskeleton (Ben-Ze'ev *et al.*, 1981; Bonneau *et al.*, 1985; Jones and Kilpatrick, 1988).

mRNA attachment to the cytoskeleton has been shown to be involved in several aspects of mRNA regulation. There is evidence to suggest that cytoskeletal interactions are required for mRNA localization (e.g., Pondel and King, 1988; Yisraeli *et al.*, 1990; Sundell and Singer, 1991; Ainger *et al.*, 1993; Ferrandon *et al.*, 1994; Pokrywka and Stephenson, 1995; Olink-Coux and Hollenbeck, 1996). In addition, it has been shown in several experimental systems that one of the requirements for translation is the attachment of RNA to the cytoskeleton. For example, in certain virus-infected cells, the replacement of host protein synthesis by viral protein synthesis is accompanied by the dis-

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placement of host RNAs from the cytoskeleton and their replacement by viral RNAs (Lenk and Penman, 1979; Van Venrooij *et al.*, 1981). In sea urchin oocytes, the stored maternal mRNAs appear to become attached to the cytoskeleton only when they become activated for translation (Moon *et al.*, 1983). It is possible that the structure provided by the cytoskeleton allows translational components and RNA to reside in close proximity, thereby increasing efficiency of translation (Negrutskii *et al.*, 1994).

Here we describe a general cytoplasmic protein complex that binds RNA and contains at least one cytoskeleton-associated protein. Using UV cross-linking we first identified a ~160-kDa protein directly bound to mRNA in vivo. Then, by electrophoretic mobility shift assays and UV cross-linking experiments in vitro, we found that a very similar, potentially identical, ~160-kDa protein is a component of a protein complex that binds RNA in a sequence-independent but size-dependent manner. In addition to the ~160-kDa protein, this ribo-protein complex contains the microtubule-associated protein, MAP 1A.

MATERIALS AND METHODS

Cell Culture and Drug Treatment

PC12 cells were grown in DMEM (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% horse serum, 5% FCS, L-glutamine (0.3 mg/ml), and penicillin/streptomycin (100 U penicillin G and 100 μg streptomycin/ml) in 5% CO_2, at 37°C. For experiments, 2×10^6 cells were plated on 100-mm poly-L-lysine–coated plates. In most experiments, cells were treated with 100 ng/ml of nerve growth factor (NGF) (Promega, Madison, WI) for 16 h. Where indicated, cells were treated with NGF for 7 d. In these cases, 2×10^5 cells were plated per dish, and media and NGF were replaced twice during this period.

In some experiments, cells were treated for 1 h with puromycin (Sigma Chemical, St. Louis, MO) at 12.5 μg/ml. The effectiveness of the drug treatment was determined by incubating control and puromycin-treated cells with ^{35}S-methionine (600 μCi/ml) (New England Nuclear, Boston, MA) for 45 min, followed by two washes with PBS. The cells were then lysed by replacing the media with 150 mM NaCl, 1% NP-40, 50 mM Tris-HCl, pH 8, at 4°C for 30 min with gentle shaking. The lysates were centrifuged at 12,000 × *g* for 10 min at 4°C, and aliquots from each supernatant were diluted 1:50 with 1 M NaOH/2% H_2O_2. The samples were then vortexed and incubated at 37°C for 10 min. To precipitate proteins, samples were diluted 1:5 with 25% trichloroacetic acid (TCA)/2% casamino acids and incubated at 4°C for 30 min. The samples were then vacuum filtered onto glass fiber filters, washed extensively with cold, 5% TCA, and given a final wash with acetone. Dried filters were counted in a scintillation counter.

In Vivo UV Cross-linking of RNA to Proteins

In vivo UV cross-linking was carried out essentially as described in Piñol-Roma *et al.*, 1989. PC12 cells were plated in 100 mm tissue culture dishes and then treated with 100 ng/ml NGF in DMEM for 16 h. The media was removed and replaced with DMEM containing 100 ng/ml NGF and ^{3}H-uridine at a final concentration of 175 μCi/ml [5,6-^{3}H]-uridine, 40 Ci/mmol; NEN, Boston, MA) for 3 h at 37°C. Puromycin was then added to a final concentration of 200 μg/ml and incubated for an additional 30 min at 37°C. The plates were washed twice with PBS. PBS (2 ml) was added to the plates, and the plates were placed at a distance of 2.5 cm from a germicidal UV light (intensity 2200 $\mu W/cm^2$) for 7 min. Protease inhibitors were added to all buffer solutions at the following concentrations: 10 mM aminoethylbenzenesulfonylfluoride (Calbiochem, San Diego, CA), 10 mM leupeptin (Sigma), 10 mM aprotinin, 1 mM pepstatin in addition to 2.5–3 U RNAse inhibitor Inhibit-ACE (5 Prime → 3 Prime, Boulder, CO). The cells were hypotonically lysed and homogenized by passing them four times through a 26-gauge needle. The nuclei were pelleted and the supernatant was heated to 65°C for 10 min and then adjusted to 1% β-mercaptoethanol, 10 mM EDTA, and 0.5 M LiCl for binding to the oligo dT cellulose. After binding at room temperature for 45 min with gentle agitation, the oligo dT was washed twice with binding buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 0.5 M LiCl, and 0.5% SDS). The poly A^+ RNA along with its covalently linked RNA-binding proteins were eluted from the oligo dT cellulose by washing twice with low-salt elution buffer (10 mM Tris, pH 7.5, 1 mM EDTA, and 0.5% SDS) containing RNAse A (final concentration of 300 μg/ml) and RNAse T1 (final concentration of 10 U/ml) for 1 h at room temperature. The eluted samples were concentrated using a Centricon 30 microconcentrator (Amicon, Beverly, MA) and then analyzed on a 10% SDS polyacrylamide gel. After electrophoresis, the gels were treated with EN ^{3}HANCE (New England Nuclear, Boston, MA), dried, and visualized by fluorography.

Preparation of Binding Extracts

PC12 and COS-7 cells were plated and grown 16 h before harvesting. PC12 cells were treated with 100 ng/ml NGF during the 16 h of growth. Tissues were handled in essentially the same manner as the PC12 and COS-7 cells with the exception that the tissues were minced and mechanically homogenized (500 rpm) before cells were pelleted and incubated in hypotonic lysis buffer. Cytoplasmic extracts were prepared essentially according to Dignam *et al.* (1983) with the exception of an incubation in high-salt (700 mM KCl) buffer prior to final centrifugation. Protease inhibitors were added to preparation buffers at the following concentrations: 10 mM aminoethylbenzenesulfonylfluoride (Calbiochem, San Diego, CA), 10 mM leupeptin (Sigma Chemical), 10 mM aprotinin (Sigma Chemical), 1 mM pepstatin (Sigma Chemical), 0.5 mM EDTA, and 1 mM DTT. Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) was used to determine the protein concentration of each lysate.

Preparation of Radiolabeled Probes and Nonradiolabeled Competitor RNA

The full-length GAP-43 cDNA (kindly provided by L. Benowitz) was used as a template in the PCR to generate DNA fragments that encompass various regions of the rat GAP-43 mRNA. Primers contained sequences for either an *Apa*I or *Nsi*I restriction site to facilitate cloning into the RNA synthesis vector, pGEM 7 (Promega, Madison, WI). The GAP-43 sequence numbers that define the DNA region of interest are as follows (numbering according to (Basi *et al.*, 1987): GAP A, 708–839, GAP B, 868-1000, and GAP C, 1023–1152. Due to errors in the PCR, there was a loss of 21 nucleotides (nt) near the 3′-end of the GAP C fragment. pGEM 7 plasmids containing either GAP A, GAP B, or GAP C DNA fragments were linearized with *Apa*I, and RNA was synthesized with SP6 RNA polymerase in the presence of ^{32}P-UTP (final specific activity, 27 Ci/mmol). Because there are 20 nt of vector sequence that contribute to each RNA probe synthesized, the length of the GAP A probe is 152 nt while the GAP B probe length is 153 nt and the GAP C probe length is 129 nt. Antisense probes were generated by linearizing the plasmids with *Nsi*I and using T7 RNA polymerase for RNA synthesis.

For preparation of the gap a, gap b, and gap c probes, complementary oligomers representing the sense or antisense strands of different regions of the rat GAP-43 cDNA were hybridized and the double-stranded DNA fragments were cloned into pGEM 7. The

gap a, gap b, and gap c regions span sequence numbers 748–775, 881–908, and 1051–1078, respectively. Because there are 20 nt of vector sequence and 6 nt of an added restriction site, when the gap a, gap b, and gap c RNA probes are synthesized, the lengths are each 54 nt.

After RNA synthesis the reaction is electrophoresed on a 6% urea polyacrylamide gel, visualized by autoradiography, and the band containing the RNA excised from the gel. The RNA is eluted from the gel slice by incubating either 4 h at 37°C or overnight at room temperature in 0.5 M ammonium acetate in diethylpyrocarbonate-treated water, pH 7.5. After centrifugation, the supernatant is removed, ammonium acetate is added to a final concentration of 2 M, and the supernatant is precipitated with 2.5 volumes of ethanol. The precipitated RNA is resuspended in diethylpyrocarbonate-treated water and an aliquot quantitated by liquid scintillation counting.

Competitor RNAs were synthesized from the same vectors as the ^{32}P-radiolabeled probes. RNA is radiolabeled for quantification by including trace amounts of ^{3}H-UTP (final specific activity, 17.5 mCi/mmol) in the RNA synthesis reaction. The competitor RNAs are gel purified as the ^{32}P-radiolabeled probes (above) with the exception that visualization after PAGE is achieved by UV shadowing against an autoradiographic intensifying screen instead of autoradiography.

Binding Reaction and Mobility Shift Assays

Radiolabeled RNA (50,000 dpm) (~240 pg of the 120- to 150-nt probes and 81 pg of the 50-nt probes) was heated to 95°C for 4 min and then cooled on ice for at least 3 min. The denatured RNA was then added to 1 μg PC12 cell extract or 10 μg tissue extract in 9 μl of 10 mM Tris, pH 7.5, 5% glycerol, 1 mM EDTA, 100 mM KCl (or greater KCl concentration when testing complex stability), and 1 mM DTT in a final volume of 10 μl. The radiolabeled RNA and proteins were incubated at room temperature for 30 min. After 50 μg of heparin were added, the incubation was continued for 10 min, and 0.5 μl of 0.1% bromophenol blue was then added. The reaction was loaded on a 6% nondenaturing polyacrylamide gel (28.2:0.8) in 0.5× TBE (1× TBE = 89 mM Tris, 8.9 mM borate, 2 mM EDTA, pH 8.3), which had been prerun overnight at 50 V and electrophoresed at 200 V for 3.5 h. The gel was dried and then visualized by autoradiography. In competition experiments 5×10^{-15} mol RNA probe (50,000–80,000 dpm of 120- to 150-nt probes or 12,000–25,000 dpm for 50-nt probes) was used and processed as described above.

Binding Reaction for Competition Assays

Binding reactions for competition assays were performed as described with the exception that the tissue extract was preincubated at room temperature for 15 min with the nonradiolabeled competitor RNA before the radiolabeled probe was added to the reaction. The random RNA polymers polyadenylic-guanylic-uridylic acid (~300 nt average length) and polycytidylic-inosinic-uridylic acid (~300 nt average length) (Sigma) were used as the single-stranded competitors and polyadenylic-polyuridilic acid (~900 bp average length) and polycytidylic-polyguanylic acid (~240 bp average length) (Sigma) were used as the double-stranded competitors. Single stranded calf thymus DNA (Sigma) (~700 nt average length) was used as the DNA competitior.

Binding Reaction for Supershift Assays

The supershift assay was performed essentially as described above with the following additions: 0.5 U of the RNAse inhibitor Inhibit-ACE (5 Prime → 3 Prime, Boulder, CO) and antibodies, with the exception of antibody clone MAP1A1, were added to the protein extract and allowed to incubate for 15 min on ice before the addition of the radiolabeled probe. The antibody clone MAP1A1 was incubated with the extract for 15 min on ice after the binding reaction and before the addition of heparin. Antibodies were added in two different forms: all were tested as ascites fluid at a dilution of 1:25, and some were also tested using 1.5 μg of the IgG fraction isolated from ascites fluid.

Antibodies

The ascites fluids used were as follows: MAP-1A (clone HM-1, Chemicon International, Temecula, CA), MAP 1A (clone 1A1, gift from G. Bloom and R. Vallee); MAP-2 (clone HM-2, Sigma), and MAP-1B (clone 3G5, Chemicon International), actin (clone C4, Chemicon International), and β-tubulin (clone TUB 2.1, Sigma). When used, the IgG fraction was isolated from ascites fluid using the E-Z-SEP IgG Partitioning Reagents (Pharmacia, Uppsala, Sweden).

In Situ UV Cross-linking and Two-Dimensional Electrophoresis

UV cross-linking was carried out essentially as described by Garcia-Blanco *et al.* (1989). After the RNA-protein binding reaction was subjected to nondenaturing PAGE as described above, the wet polyacrylamide gel was placed on ice at a distance of 8 cm from a germicidal UV light (intensity 2200 μW/cm^2) for 20 min. After UV cross-linking, the polyacrylamide gel was wrapped in Saran wrap and visualized by autoradiography at 4°C overnight. The lane containing the RNA–protein complexes was excised, placed in a tube with TE (10 mM Tris, pH 8, and 1 mM EDTA) containing 330 μg/ml RNAse A and 50 U/ml RNAse T1 and incubated at 37°C for 1 h. The RNase solution was removed, and the gel slice was incubated with 2 × SDS PAGE buffer at 37°C for 1 h and subsequently at 65°C for 15 min. The gel slice was embedded into the stacking portion (6 cm) of a 10% SDS-polyacrylamide gel (17 cm separating) by layering low melting temperature agarose below and above the gel slice to facilitate the embedding.

Electroelution of Complex 1 Protein Components

Complex 1 was excised from a UV cross-linked nondenaturing polyacrylamide gel as described above. The gel slice was placed into the Hoefer GE 200 SixPac Gel Eluter (Hoefer, San Francisco, CA), and the proteins were eluted by applying 50 V for 200 min. The eluate was treated for 30 min at room temperature with Rnase A and RNase T1 at concentrations of 100 μg/ml and 50 U/ml, respectively. The proteins were precipitated in the presence of 25 μg/ml BSA carrier protein by addition of TCA to a final concentration of 5%. The precipitate was washed, resuspended in 1× Laemmli loading buffer and analyzed by SDS-PAGE.

Peptide Analysis of the in Vitro Identified ~160-kDa RNA-Binding Protein

Radiolabeled GAP A (2×10^6 dpm) was incubated with 375 μg of brain extract, electrophoresed through a nondenaturing polyacrylamide gel, UV cross-linked, and visualized as described above. The region of complex 1 was excised, treated with RNase, and equilibrated in SDS loading buffer as above. After electrophoresis through a 10% denaturing polyacrylamide gel, the region containing 160-kDa proteins was excised, and the slice was equilibrated in denaturing buffer as described by Cleveland *et al.* (1977). The slice was embedded into the stacking portion (6 cm) of a 15% SDS polyacrylamide gel (17 cm separating) by layering low-melting-temperature agarose in the well below the slice, to facilitate embedding. Digestion was carried out essentially as described by Cleveland *et al.* (1977). Briefly, the gel slice was overlayed with 50 μg of *Staphylococcus aureus* V8 protease (Sigma) in 20 μl 0.125 M Tris, pH 6.8, 0.1% SDS, 1 mM EDTA, 20% glycerol, and 0.005% bromophenol blue. Electrophoresis proceeded until the bromophenol blue was within the last centimeter of the stacking gel. At that time, the power was

turned off for 45 min, after which electrophoresis continued until the bromophenol blue was near the bottom of the separating gel.

Peptide Analysis of the in Vivo Identified ~160-kDa mRNA-binding Protein

In vivo cross-linking of PC12 cells was carried out as described above. After electrophoresis, the region containing 160-kDa proteins was excised and the gel slice was equilibrated in denaturing buffer as described by Cleveland *et al.* (1977). The slice was subsequently processed and digested with *S. aureus* V8 protease as described above for the in vitro peptide analysis.

RESULTS

In Vivo Evidence for a ~160-kDa mRNA-binding Protein

To identify general RNA–protein interactions in the cytoplasm, PC12 cells were radiolabeled with tritiated uridine and exposed to UV light to cross-link all of the cellular RNAs to the proteins with which they are interacting in the living cell. A number of proteins, both nuclear and cytoplasmic, have been previously shown to be complexed with mRNA in vivo by means of such UV cross-linking (Piñol-Roma *et al.*, 1989). To exclude RNA–protein interactions related to protein synthesis, the cells were first treated with a concentration of puromycin that inhibited translation by 97%, as assessed by incorporation of ^{35}S-methionine (our unpublished observations; see MATERIALS AND METHODS). The cells were then exposed to UV light to cross-link the cellular RNAs to their associated proteins. Oligo-dT cellulose was used to isolate total poly A^+ RNA along with its cross-linked proteins from the cells. After binding the oligo dT cellulose, the proteins that were covalently linked to the poly A^+ RNA were recovered by low-salt and RNAse A and RNAse T1 treatment. The released proteins, along with the few remaining covalently linked radionucleotides, were then subjected to SDS-PAGE and detected by fluorography. Several radiolabeled proteins, including an approximately 160-kDa species, were observed (Figure 1).

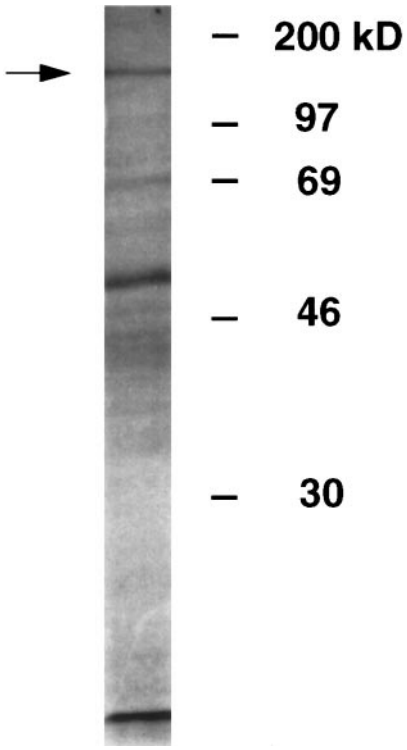

Figure 1. Poly A^+ RNA is bound by a ~160-kDa protein in vivo. NGF-treated PC12 cells were preincubated with ^{3}H-uridine and exposed to UV light. The cross-linked poly-A^+ RNA–protein complexes were isolated by oligo-dT cellulose selection. The eluted radiolabeled protein species were resolved on a 10% SDS polyacrylamide gel and visualized by fluorography. The arrow denotes the ~160-kDa RNA-binding protein.

The ~160-kDa RNA-binding Protein Detected in Vivo Is, or Is Closely Related to, a Component of a Protein Complex That Binds RNA in a Sequence-insensitive, Size-dependent Manner in Vitro

Due to the nature of the in vivo cross-linking method, the mRNA–protein interactions it identifies most likely represent the average of frequent events that involve most mRNA molecules in the cell. To further examine these general RNA-protein interactions, studies were carried out to identify and characterize RNA-protein interactions in an in vitro system. The electrophoretic mobility shift assay (EMSA) has been used extensively to detect the association of nucleic acids and proteins. Here EMSA was used to analyze the interaction of protein extracts and in vitro-synthesized RNA as a means of probing the nt sequence-independent association of proteins with mRNA.

Sequence Independence Protein extracts were incubated with several in vitro-synthesized, radiolabeled RNAs and analyzed by EMSA. In initial experiments we used NGF-treated PC12 cell extracts and three RNA species as probes, GAP A, GAP B, and GAP C, each encoding approximately equal portions of the 3′-untranslated region (UTR) of the GAP-43 mRNA (Figure 2A) and sharing very low levels of homology with each other. The PC12 cell protein extract contained proteins that bound each RNA and gave rise to distinct RNA-protein complexes (Figure 2B). At least one common, comigrating complex was formed independently of the RNA probe used. This common complex is the largest (least mobile) detected and will be subsequently referred to as complex 1. The generation of seemingly identical comigrating complex 1 bands using different RNAs was indicative of a general sequence-independent RNA–protein interaction. Protein extracts from brain were then also tested for their ability to interact with the different RNA species. As

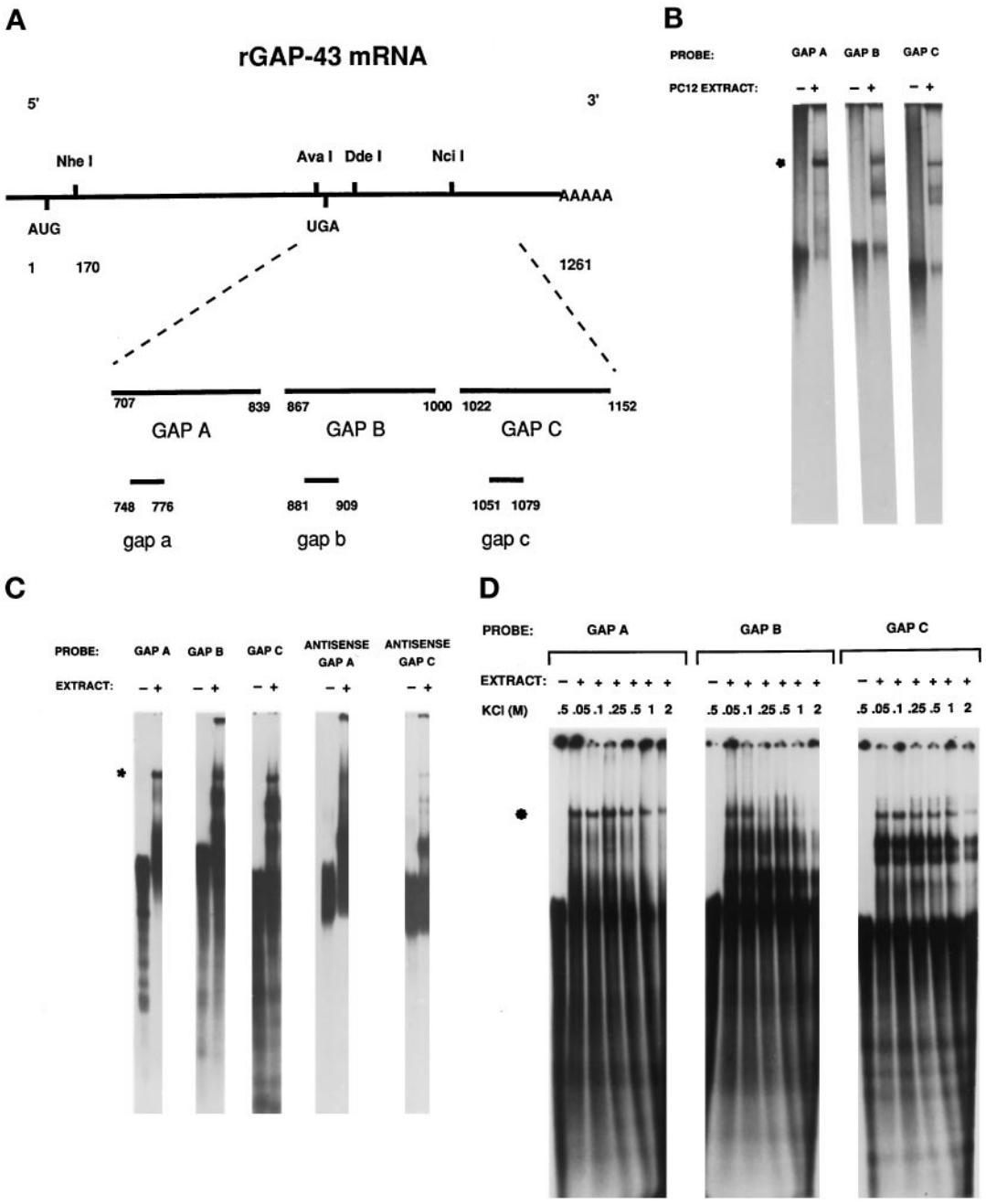

Figure 2. RNA–protein complex 1 forms with many RNA species over a range of salt concentrations. (A) The map of rat GAP-43 mRNA indicates the location of the GAP A, GAP B, GAP C, gap a, gap b, and gap c RNA probes used in RNA mobility shift assays. The numbering is according to Basi *et al.* (1987). The translational stop codon, UGA, is located at sequence number 731. (B) Radiolabeled GAP A, GAP B, and GAP C were incubated with extracts from 16-h NGF-treated PC12 cells and electophoresed through a 6% nondenaturing polyacrylamide gel. The asterisk indicates a common, comigrating RNA–protein complex (complex 1) that formed with each RNA molecule. (C) Radiolabeled GAP A, GAP B, GAP C, antisense GAP A, and antisense GAP C were incubated with brain protein extract and electrophoresed through a 6% nondenaturing polyacrylamide gel. The asterisk indicates complex 1 formed with each probe. (D) Radiolabeled GAP A, GAP B, and GAP C RNA probes were incubated with brain protein extract in the presence of increasing amounts of KCl and electrophoresed through a 6% nondenaturing polyacrylamide gel. The asterisk indicates complex 1.

with PC12 cell extracts, we observed that the brain extract could form complex 1 with GAP A, GAP B, or GAP C probes (Figure 2C). Indeed, in all subsequent experiments, brain extract and PC12 cell extract behaved in an essentially identical manner.

The above results suggest that the formation of the common RNA–protein complex 1 is due to sequence-independent recognition of RNA by proteins present in the brain and PC12 cell extracts. As a further test of the sequence-independent nature of complex 1, antisense GAP A and antisense GAP C probes were generated and used in mobility shift experiments. As with GAP A, GAP B, and GAP C probes, complex 1 was formed when brain extracts were incubated with the antisense RNA probes (Figure 2C). Indeed, several other RNA probes of unrelated sequence and greater than 120 nt in length were also capable of complex 1 formation (our unpublished observations). To determine whether complex 1 could be formed with proteins found in other tissues, protein extracts derived from liver, lung, muscle, and testes were tested for their ability to bind to GAP A, GAP B, and GAP C as compared with brain. In each case, complex 1 formed, although to different degrees (our unpublished observations). These results indicate that proteins found in a number of tissues can bind RNA regardless of its primary sequence and suggest that complex 1 may represent an RNA–protein interaction common to many cell types.

Because some of the species detected by EMSA might represent nonspecific, ionic RNA-protein interactions, the capacity of the protein extract to interact with either the GAP A, GAP B, or GAP C probes was tested in the presence of increasing concentrations of salt. Most RNA–protein complexes formed were stable in 1 M KCl, and some were still visible in 2 M KCl (Figure 2D). In particular, complex 1 begins to destabilize when formed in 0.25 M KCl with GAP B, but it remains stable in as much as 2 M KCl when formed with GAP A or GAP C. Because probe composition appears to affect the differential stability of complex 1 in some salt conditions, it suggests that protein binding exhibits a preference, but not a requirement, for particular RNA sequences. The ability of complex 1 to form under conditions of high ionic strength suggests that hydrophobic and hydrogen bond, rather than electrostatic/ionic interactions, are most important for complex 1 stability.

The RNA sequence-independent nature of complex 1 was further investigated by competition assays. First, experiments were performed to determine the amount of protein necessary to keep complex formation in the linear range of the binding curve. Nonradiolabeled GAP A, GAP B, and GAP C RNAs were then incubated with brain protein extract and either radiolabeled GAP A, GAP B, or GAP C probes. Complex 1, formed with either the GAP A, GAP B, or GAP C probe, was competed very effectively by excess nonradiolabeled GAP A, GAP B, or GAP C RNAs although there were slight differences in the ability of the individual species to act as competitors (Figure 3A).

Experiments were also carried out in which random single-stranded RNA polymers and double-stranded RNA polymers were used to compete for binding of proteins in complex 1. A representative example using the GAP C probe shows that while single-stranded random RNA polymers were very effective competitors, double-stranded RNA polymers were not (Figure 3B). Similar competition assays were performed using random single-stranded DNA. Using both GAP A and GAP C as probes, single-stranded calf thymus DNA was unable to effectively compete the formation of complex 1 (our unpublished observations). These results provide further evidence that complex 1 formation is the result of one or several ubiquitous proteins interacting with single-stranded RNA in a sequence-independent manner. Although the proteins of complex 1 appear to have some preference, or different affinity, for different nt sequences, there is no absolute nt sequence requirement for formation of complex 1.

Size Dependence. The RNA species tested thus far in the mobility shift assays were at least 120 nt in length and were all capable of forming complex 1. To investigate the size requirement for complex 1 formation, we constructed RNA probes that were 54 nt in length and represented a subset of the larger GAP A, GAP B, and GAP C probes. Their ability to interact with brain protein extract was determined by EMSA as previously described. Use of the shorter probes, denoted gap a, gap b, and gap c, respectively (Figure 2A), resulted in the formation of RNA–protein complexes that did not comigrate with complex 1 (Figure 3C). These and results with other various sized probes (our unpublished results) indicate that the formation of complex 1 is dependent on a minimum length of RNA of approximately 120 nt.

To explore the possibility that some protein component(s) of complex 1 is (are) also included in the RNA-protein species detected by mobility shift assay using the (shorter) gap a, gap b, or gap c probes, competition experiments were performed. Nonradiolabeled gap a, gap b, or gap c was added to binding reactions of radiolabeled GAP A, GAP B, or GAP C probes and brain extract. Although gap a, gap b, and gap c do not form complex 1, each species was able to interfere with the formation of complex 1 and other complexes formed by RNA-protein interactions with the larger probes (Figure 3D). Although there were slight differences in the ability of each of the shorter gap probes to compete with the radiolabeled GAP A, GAP B, or GAP C probes for protein binding, none were as effective as the GAP A, GAP B, or GAP C competitors themselves (Figure 3A). These findings indicate that while the

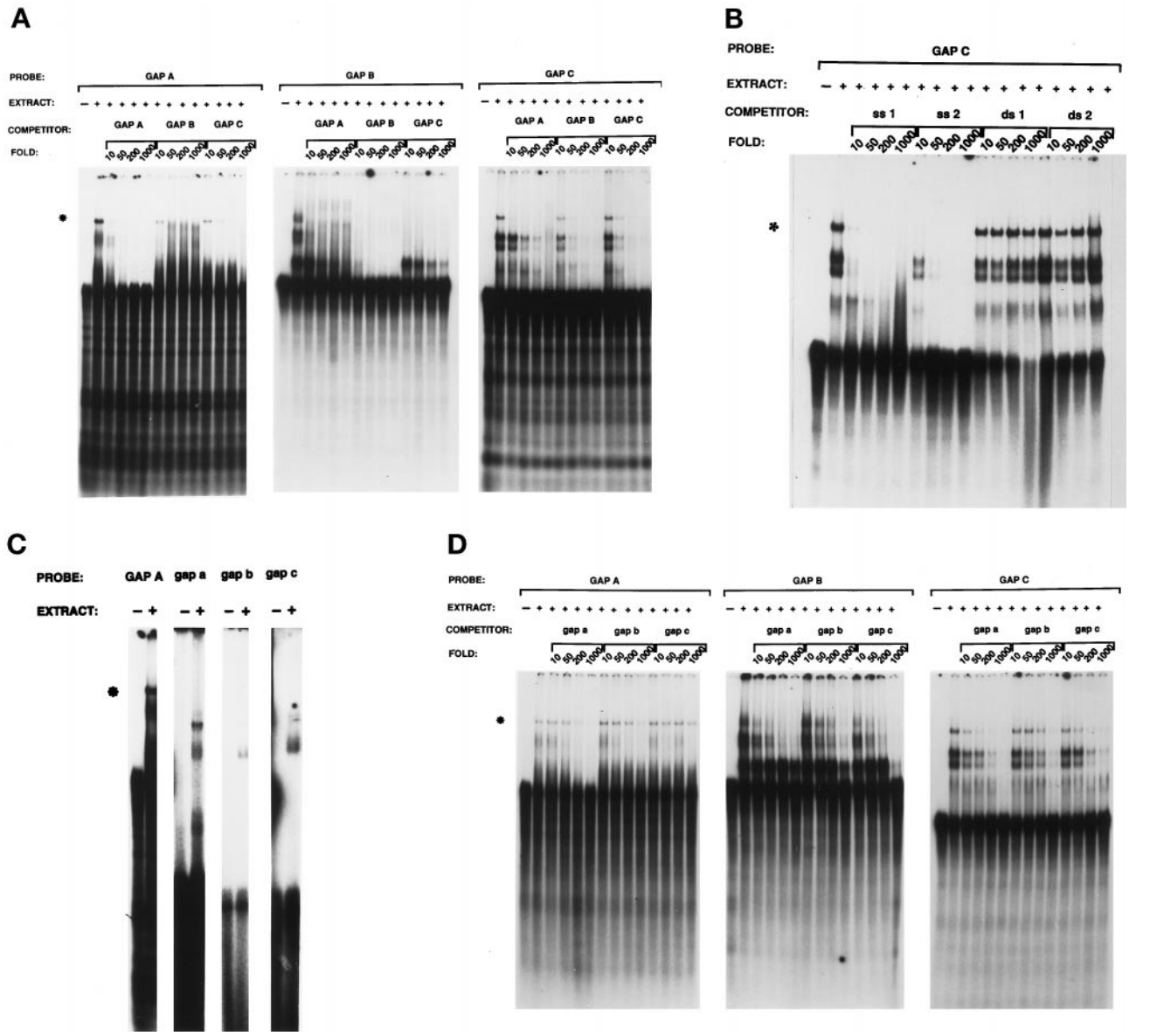

Figure 3. Complex 1 forms with single-stranded RNA in a sequence-insensitive and size-dependent manner. (A) Complex 1 forms in a relatively sequence-insensitive manner. Excess nonradiolabeled GAP A, GAP B, and GAP C were added, at the indicated molar concentrations, to binding reactions containing brain protein extract and the GAP A, GAP B, or GAP C RNA probes. (B) Complex 1 forms with single-stranded, but not double-stranded, RNA. Excess random RNA polymers were added at the indicated molar concentrations to binding reactions containing brain protein extract and the GAP C RNA probe, where ss 1 represents single stranded polyadenylic-guanylic-uridylic acid (AGU); ss 2, single-stranded polycytidylic-inosinic-uridylic acid (CIU); ds 1, double-stranded polyadenylic-polyuridylic acid (A·U); and ds 2, double-stranded polycytidylic-polyguanylic acid (C·G). (C) Complex 1 forms in a size-dependent manner. Radiolabeled GAP A, gap a, gap b, and gap c were incubated with brain protein extract and electrophoresed through a 6% nondenaturing polyacrylamide gel. Complex 1, indicated by the asterisk, is observed only with the GAP A probe. (D) Although not capable of forming complex 1, small RNAs interact with some components of complex 1 and inhibit its formation. Excess nonradiolabeled gap a, gap b, or gap c were added at the molar excess indicated to binding reactions containing brain protein extracts and GAP A, GAP B, and GAP C RNA probes. The asterisk indicates complex 1.

smaller RNAs cannot interact with the extract proteins to form complex 1, they are capable of binding at least one component found in the brain extracts that is necessary for complex 1 formation.

A ~160-kDa Protein Is Found in Complex 1 by in Vitro UV Cross-linking. If complex 1 represents a nt sequence-independent RNA–protein interaction that occurs in vivo, its RNA-binding component(s) should

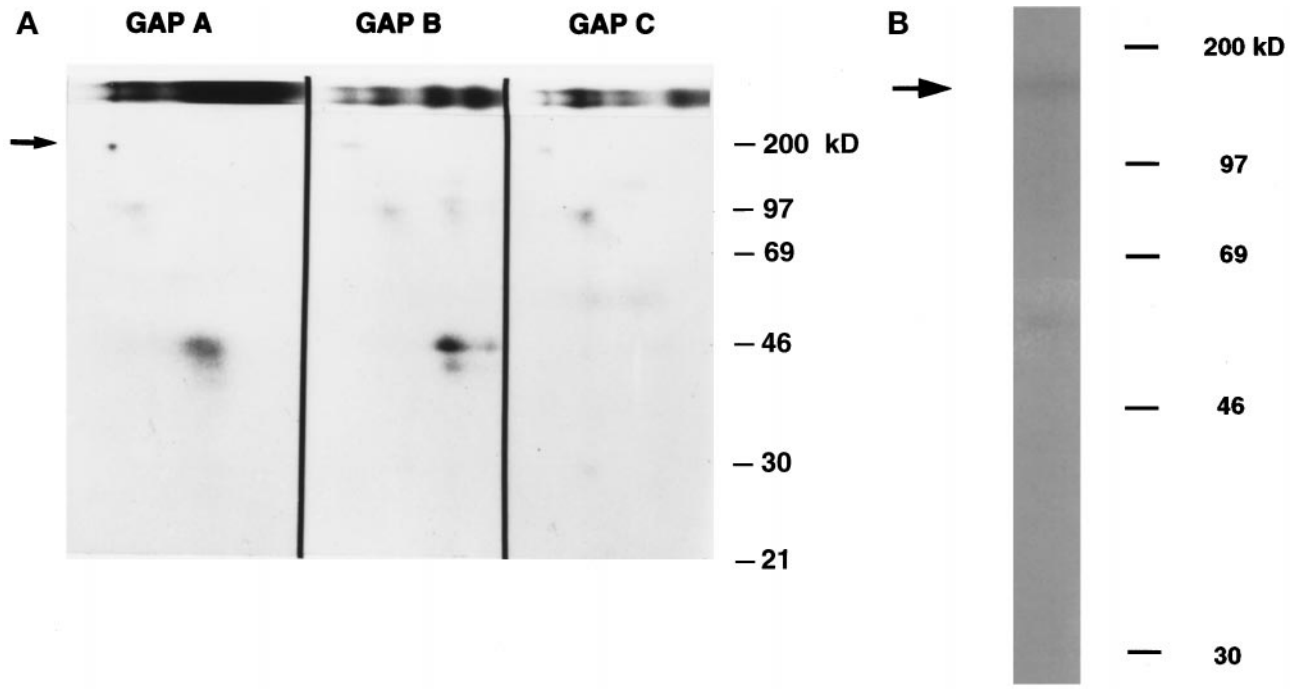

Figure 4. Complex 1 contains a ~160-kDa RNA-binding protein. (A) ^{32}P-radiolabeled GAP A, B, and C were incubated with brain protein extract and analyzed on a 6% nondenaturing polyacrylamide gel. The gel was exposed to UV light, and the protein–RNA complexes were visualized by autoradiography. The entire lane in which the reaction was run was excised and layered (as shown in the upper portion of the figure) onto a 10% SDS polyacrylamide gel. The arrow indicates a ~160-kDa protein associated with complex 1. (B) Binding reaction of GAP A probe and protein extract was electrophoresed through nondenaturing gels and visualized as in panel A. Complex 1 was excised, and the proteins were electroeluted and analyzed on a 10% SDS polyacrylamide gel. The arrow indicates a ~160-kDa protein associated with complex 1.

be similar to those found complexed to mRNA in vivo. To address this question, the protein(s) comprising complex 1 were further characterized in experiments employing in situ cross-linking and two-dimensional gel electrophoresis. After the RNA–protein binding reaction and electrophoresis for mobility shift detection, the nondenaturing polyacrylamide gel was exposed to UV light to covalently cross-link the radiolabeled RNA to proteins at sites of direct RNA-protein contact. This procedure, in effect, transfers the radiolabeled nt of the RNA to the protein. The lane from the nondenaturing polyacrylamide gel was then excised, treated with RNase to trim all but the short stretch of nt that are cross-linked to, or protected by, the protein, and layered onto a SDS-polyacrylamide gel for electrophoresis in the second dimension (Garcia-Blanco *et al.*, 1989). After electrophoresis and detection by autoradiography, the size of the protein(s) bound directly to the radiolabeled RNA could be estimated (mass determination will be approximate because migration of the RNA-binding proteins will be slightly affected by the presence of the cross-linked oligonucleotide fragment). Such in situ cross-linking of complex 1 formed with either the GAP A, GAP B, or GAP C probes and the protein extract revealed a major radiolabeled protein species of approximately 160 kDa (Figure 4A). The size of this RNA-binding protein is identical to that of one of the proteins detected by the in vivo cross-linking of RNA described earlier (Figure 1).

Other radiolabeled species were also detected with each probe. Some species formed with GAP A, GAP B, and GAP C comigrated and may represent other sequence-independent binding proteins, while species that were unique to each probe may represent sequence-specific binding proteins.

The presence of the ~160-kDa protein in complex 1 was confirmed by excising complex 1 from a nondenaturing gel that had been UV cross-linked as described above. After RNase treatment of excised complex 1, the associated proteins were electroeluted and analyzed by SDS-PAGE (Figure 4B). In addition to a ~160- kDa RNA-binding protein, a ~60-kDa protein species was observed. Because this species was not evident as a component of complex 1 by the two-dimensional cross-linking analysis (Figure 4A), it likely represents a degradation product of the ~160-kDa RNA-binding protein.

In situ cross-linking experiments as described above were also performed using the 54-nt gap RNA as probe. A ~160-kDa protein did not appear to be cross-linked to this probe (our unpublished results), thus indicating that the formation of complex 1 is not

merely due to cooperative binding and stabilization of a protein complex that also binds shorter RNA probes.

V8 Protease Digestion Indicates That the ~160-kDa mRNA-binding Protein Observed in Vivo Is Highly Similar to the ~160-kDa Protein Identified in Vitro. Thus far, two RNA-binding proteins have been described: a ~160-kDa protein that interacts with RNA in a sequence-independent, size-dependent manner in vitro, and a ~160-kDa protein that cross-links to the majority of poly A^+ RNA in vivo. To determine whether these general RNA-binding proteins were related, we cross-linked proteins to radiolabeled RNA both in vivo and in vitro and compared their radiolabeled peptide profiles after digestion by S. aureus V8 protease. For analysis of the RNA-binding protein found in vitro, EMSA was performed using the protein extract and the ^{32}P-radiolabelled GAP A probe. After UV cross-linking and autoradiographic visualization, complex 1 was excised from the nondenaturing polyacrylamide gel, treated with RNAse A and RNAse T1, and electrophoresed on a denaturing polyacrylamide gel as described above. The region of the ~160-kDa protein was then excised and layered onto the stacking gel of a denaturing polyacrylamide gel along with *S. aureus* V8 protease. Digestion by *S. aureus* V8 protease occurred in situ during a pause in electrophoresis through the stacking gel (Cleveland *et al.*, 1977). Two major radiolabeled peptide fragment species, approximately ~7 kDa and ~9 kDa, remained after digestion (Figure 5).

In vivo RNA-protein cross-linking experiments were performed using PC12 cells grown in tritiated uridine. Cross-linked mRNA-binding proteins were bound and then eluted from oligo-dT cellulose as previously described. After electrophoresis on a denaturing polyacrylamide gel, the region of the ~160-kDa protein was processed as described for the ~160-kDa species observed in vitro. Similar ~7-kDa and ~9-kDa radiolabeled peptide fragments remained after V8 digestion (Figure 5).

Because the radiolabeled probes were quite different in the in vitro and in vivo analysis of the 160-kDa protein, and therefore the relative abundance of U nucleotides in the portions of the probe cross-linked to and protected by the 160-kDa protein, the amount of radioactivity in the two V8 digestion fragments would not be expected to be the same. Specifically, the in vitro experiment used a probe of known sequence from the GAP43 3′-UTR that has a particular concentration of U nucleotides (GAP-A). In contrast, the in vivo experiment per force analyzed the cross-linking of the 160-kDa species to all intracellular mRNAs, which would represent a random collection of sequences. As a result, we would not expect the relative intensities of the two cross-linked species after V8 digestion to be the same, and they were not. However, the relative sizes of the species derived from the in vivo and in vitro experiments were identical, suggesting that the original 160-kDa proteins were identical, or highly similar. Proof of their identity must await biochemical purification of the intact RNA-binding complex from both in vivo and in vitro binding reactions, purification of the two 160-kDa proteins from the other components of the complex, and microsequencing.

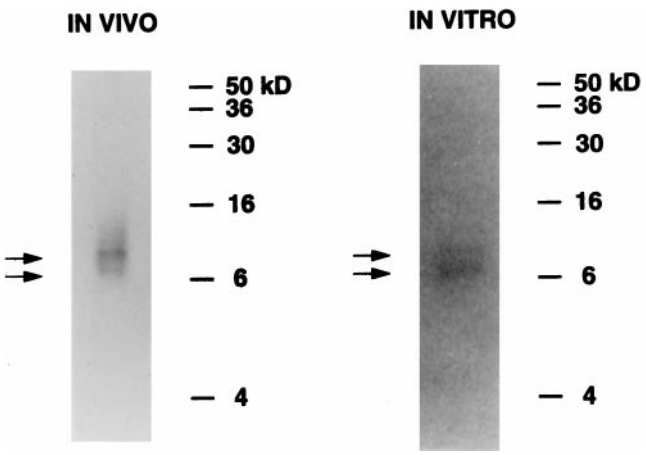

Figure 5. V8 digestion indicates that the ~160-kDa RNA-binding proteins identified in vivo and in vitro are highly similar. The ~160-kDa protein was excised from the 10% SDS polyacrylamide gels used to separate the UV cross-linked RNA-binding proteins in both the in vivo and the in vitro experiments (as described in Figures 1 and 4A, respectively). The gel slice was layered onto the stacking gel of a 17.5% SDS polyacrylamide gel along with *S. aureus* V8 protease. Digestion by *S. aureus* V8 protease occurred in situ during a pause in electrophoresis through the stacking gel. Radiolabeled peptide fragments ~7 kDa and ~9 kDa (arrows) were obtained from V8 digestion of both the ~160-kDa RNA-binding proteins found in vivo and in vitro. As explained in the text, the fact that the radiolabeled RNA probes used for the in vivo experiment (total cellular mRNA) and the corresponding in vitro experiment (GAP A) are different makes it unlikely that the number of radioactive U nucleotides in the RNA fragments cross-linked to the two V8 digestion fragments would be equal in the two experiments. Only the equal size of the fragments is relevant to the question of whether the two 160-kDa proteins are similar.

In both the in vitro and in vivo experiments the visualization of the ~160-kDa protein species, as well as the resultant peptide fragments, is dependent on the ability of the ~160-kDa protein to form covalent adducts with radiolabeled ribonucleotides. This criterion restricts the identity of the ~160-kDa protein to a functional RNA-binding protein. Because there is a limited number of ~160-kDa proteins that can bind RNA and fulfill the additional requirement that its digestion by a given amount of *S. aureus* V8 protease yields both a 7- and a 9-kDa peptide fragment bound to a ribonucleotide fragment, it is most likely that the ~160-kDa protein detected in vitro as part of complex 1 is identical or very similar to the ~160-kDa protein detected by in vivo cross-linking. These results indicate that our in vitro findings accurately reflect the properties of a general mRNP present in the cell cytoplasm.

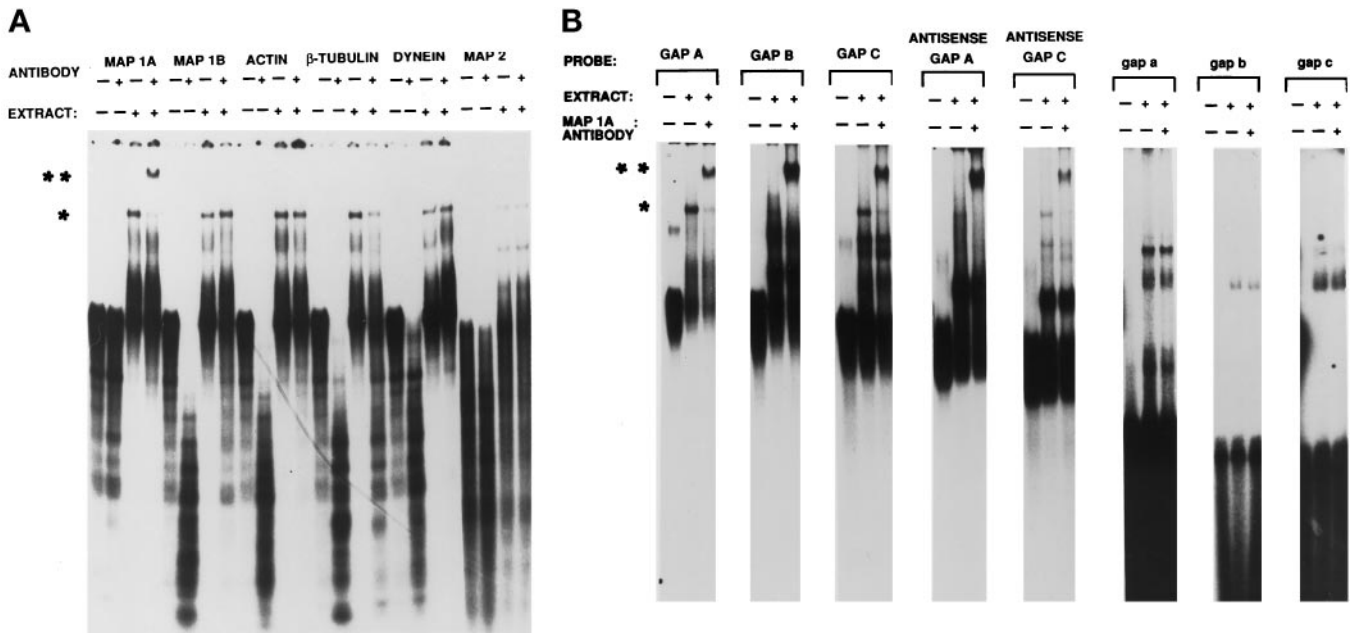

Figure 6. The microtubule-associated protein MAP 1 A is a component of complex 1. (A) Radiolabeled GAP A was incubated with brain protein extract in the presence of antibodies to several cytoskeletal components and electrophoresed through a 6% nondenaturing polyacrylamide gel. For each probe, control lanes with probe alone, probe with antibody alone, and probe with extract alone are shown to the left of the experimental lanes containing probe, extract, and antibody. The single asterisk indicates the position of complex 1, and the double asterisk indicates the position of the supershifted complex 1. (B) GAP A, GAP B, GAP C, antisense GAP A, antisense GAP C, gap a, gap b, and gap c RNA probes were incubated with brain protein extract in the presence of antibodies that recognize MAP 1A. The first two lanes for each of the gap a, gap b, and gap c probes are identical to those shown in Figure 3c. The single asterisk denotes complex 1, while the double asterisk denotes the supershifted complex 1, indicating the presence of MAP 1A.

The RNA-binding Complex 1 Includes the Cytoskeletal Associated Protein MAP 1A

The sequence-independent nature of RNA binding by the ~160-kDa protein of complex 1 and its apparently general association to mRNA in vivo is consistent with the possibility that it is involved in binding mRNA to the cytoskeleton. To test this possibility, the presence of cytoskeletal components in the in vitro-formed complex 1 was monitored using the "supershift" assay. In this assay antibodies to possible protein components of the RNA–protein complex are included in the reaction mixture containing the protein extract and the RNA probe. If the antibody binds a component of a complex, complex formation can be inhibited or the migration of the resultant complex can be retarded (supershifted) as compared with the original complex detected in standard EMSA. Among a number of antibodies directed toward cytoskeletal elements, only antibodies to MAP 1A (HM1 clone) (Huber and Matus, 1984) decreased the mobility of, or supershifted, complex 1 (Figure 6A). MAP 1A, whose function remains unknown, is one of the major proteins found associated with microtubules and is widely distributed among different cell types (Bloom *et al.*, 1984; Wiche *et al.*, 1984). A second antibody to MAP 1A (MAP 1 A1 clone) (Bloom *et al.*, 1984) was capable of disrupting complex 1, strengthening the conclusion that complex 1 contains MAP 1A, rather than an unknown MAP 1A cross-reacting species (our unpublished results). Complex 1 was neither supershifted nor disrupted with antibodies to MAP 2, β-actin, β-tubulin, tau, or cytoplasmic dynein (Figure 6A). In all cases the antibody added to the probe alone had no effect on its mobility. These results suggest that MAP 1A is a component of a protein complex that binds to single-stranded RNA in a sequence-independent but size-dependent manner.

To determine whether MAP 1A was a general component of complex 1 formed with different RNA probes, we tested the ability of complex 1 formed with GAP B, GAP C, antisense GAP A, and antisense GAP C probes to interact with antibodies to MAP 1A. As with GAP A, the complex 1 formed with each of the different RNA probes was supershifted by the MAP 1A antibodies (Figure 6B). Moreover, the addition of the MAP 1A antibodies often resulted in the stabilization of complex 1 as indicated by the increase in intensity of the supershifted band as compared with the original complex 1 band. MAP 1A antibodies did not affect the migration of any of the complexes formed with gap a, gap b, or gap c probes, indicating the specificity of the complex 1–anti-MAP 1A interaction

(Figure 6B). Evidently, only complex 1, capable of forming only with longer RNA probes, includes the cytoskeletal element MAP 1A.

DISCUSSION

The close association of mRNA with the cytoskeleton of eukaryotic cells has been a recurring and increasingly convincing finding in a number of experimental systems. The full physiological significance of the general association of mRNA with the cytoskeleton is not yet known, but there is evidence to suggest that cytoskeletal interactions are important for expression of mRNA, including control of its translation and its directed movement within the cell. In many cell types (Wilhelm and Vale, 1993) including neurons (Chicurel and Harris, 1992; Steward and Banker, 1992; Chicurel *et al.*, 1993) localization of mRNA to specific subcellular compartments is a potentially important regulatory step in the synthesis of proteins (for review see Steward, 1994, and St Johnston, 1995).

The cytoplasm is a viscoelastic gel network that acts as a barrier to the free diffusion of macromolecules (Luby-Phelps, 1993). However, the cytoskeletal network provides both a railroad and a scaffold on which RNA can be efficiently translocated and anchored within the cell. To facilitate movement along the cytoskeleton and perhaps regulate its expression, it is likely that RNA is packaged into compact particles. Interestingly, transported mRNAs have been microscopically visualized as particles that represent either ribonucleoprotein complexes or vesicles (Ainger *et al.*, 1993; Ferrandon *et al.*, 1994; Wang and Hazelrigg, 1994; Knowles *et al.*, 1996). The movement of such mRNA-containing particles is apparently an active process (Ainger *et al.*, 1993; Knowles *et al.*, 1996).

Since the vast majority of mRNAs are associated with the cytoskeleton, it seems reasonable that a feature common to all mRNAs is responsible for the linkage. Thus it has been suggested that the 5′-cap structure (Zumbe *et al.*, 1982) or the poly A tail (Taneja *et al.*, 1992) may direct the mRNA–cytoskeleton interaction. However, RNAs such as poliovirus that lack a 5′-cap (Bonneau *et al.*, 1985) and histone and reovirus RNA that lack a poly A tail (Jeffery *et al.*, 1983; Bonneau *et al.*, 1985; Zambetti *et al.*, 1985) are also found associated with the cytoskeleton. This indicates that the cap-binding protein (Filipowicz *et al.*, 1976; Sonenberg *et al.*, 1979) or the poly A-binding protein (Van Venrooij *et al.*, 1977) cannot alone account for binding mRNA to the cytoskeleton. Instead, we considered that a protein complex that binds RNA in a sequence-independent manner is a more likely candidate for linking RNA to the cytoskeleton.

Until now, proteins involved in the general attachment of RNA to the cytoskeleton have not been identified. Here we describe a ~160-kDa protein that is bound directly to mRNA in vivo and a similar and possibly identical protein that can bind RNA in vitro. The ~160-kDa protein is present in vitro in an RNA–protein complex that includes the microtubule-binding protein MAP 1A. This RNA–protein complex is resistant to dissociation by high salt, requires a minimum length of single-stranded RNA of approximately 120 nt, and forms in a relatively nt sequence-independent manner. Possible roles of this complex include localizing and anchoring mRNA, packaging mRNA, and regulating mRNA stability or translation.

If the ~160-kDa protein interacts with RNA by binding at many sites within each mRNA molecule, it would resemble several other proteins and protein complexes that bind nucleic acids in a sequence-independent manner. In addition to the DNA-binding nucleosome, a similar RNA-binding particle—the ribonucleosome—has been reported to package nuclear RNA. These RNP particles have been isolated, analyzed, and found to be composed of heterogeneous nuclear (hnRNP) proteins (for reviews, see Beyer and Osheim, 1990; Dreyfuss *et al.*, 1990). Thus, in additon to their role in splicing (Gorlach *et al.*, 1993), the isolated hnRNP proteins are capable of binding RNA in a sequence-independent and size-dependent manner, similar to the cytoplasmic RNA-binding complex that we have identified. It is therefore possible that in a manner analogous to hnRNP binding to hnRNA, the ~160-kDa protein may play some role in "packaging" mRNA in the cytoplasm, a putative "cytosome" (Chicurel *et al.*, 1996).

Whether such a cytosome complex is only involved in anchoring the RNA to the cytoskeleton or may also participate in transport of the RNA is currently unknown. Indeed, there is at least one example in which nucleic acid movement requires two different protein complexes to carry out the separable functions of attachment and movement. Chromosome translocation along spindle microtubules apparently requires both the microtubule motor-containing complex, CBF3, and other protein(s) whose sole function is to attach the chromosomes to the microtubules (Sorger *et al.*, 1995).

In addition to their effect on mRNA movement and spatial distribution, mRNA–cytoskeletal interactions likely play a role in regulating translation. Evidence suggests that protein synthesis in mammalian cells occurs in a "channeled pathway," in which many of the components of the synthetic machinery are spatially restricted, such that individual components are directly transferred to the translational complex without dissociating into the soluble phase of the cytoplasm (Negrutskii *et al.*, 1994). In accordance with these findings, a number of proteins that are involved in translational regulation, including the cap-binding protein (Zumbe *et al.*, 1982), translation initiation factors eIF-2, eIF3, eIF4A, and eIF-5 (Howe and Hershey, 1984; Gavrilova *et al.*, 1987; Heuijerjans *et al.*, 1989),

and elongation factors eEF1α (Yang *et al*., 1990) and eEF1–2 (Gavrilova *et al*., 1987) have been found associated with the cytoskeleton. Other proteins involved in posttranscriptional regulation such as poly A^+-binding protein (Greenberg, 1980) and seryl-tRNA synthetase (Miseta *et al*., 1991) can be found UV cross-linked to the mRNA in vivo. The RNA–protein complex 1 described here, which includes components that bind mRNA directly and others that are associated with the cytoskeleton, may similarly participate in the regulation of translation.

Our finding of an in vitro interaction between a MAP 1A-containing protein complex and RNA would seem to implicate the microtubule network as the cytoskeletal system to which the RNA is attached. Interestingly, in the dendrites of neuronal cells, mRNA is preferentially found between, rather than on, microtubules (Bassell *et al*., 1994), as is MAP 1A (Shiomura and Hirokawa, 1987). This is consistent with our findings of the association of mRNA with the microtubule-associated protein MAP 1A which, as a microtubule cross-linker, would be found between microtubules. However, there is evidence indicating that MAP 1A is not exclusively associated with microtubules but may also be associated with other, as yet unidentified, intracellular structures (Asai *et al*., 1985; Herrmann and Wiche, 1987; Fujii *et al*., 1993). Thus, complex 1 may mediate attachment of RNA to microtubules, and/or other filament systems. Indeed, in other experiments, microfilaments (e.g., Ornelles *et al*., 1986; Yisraeli *et al*., 1990; Taneja *et al*., 1992), intermediate filaments (e.g., Pondel and King, 1988), and microtubules (e.g., Raff *et al*., 1990; Yisraeli *et al*., 1990; Ainger *et al*., 1993; Ferrandon *et al*., 1994; Litman *et al*., 1994; Pokrywka and Stephenson, 1995; Olink-Coux and Hollenbeck, 1996) have all been implicated in the association of mRNA with the cytoskeleton. Interestingly, while this manuscriprt was being reviewed, MAP 1A light chain 3 was reported to bind to an AU-rich region in the fibronectin mRNA 3′-UTR, and to enhance its translation (Zhou *et al*., 1997), further implicating this protein in mRNA physiology.

Taken together, our data suggest that RNA associates with the cytoskeleton through a protein complex that includes MAP 1A and a ~160-kDa protein that binds directly to RNA. The general association of mRNA with the cytoskeleton may play an important role in controlling mRNA expression by regulating more specific interactions, such as those involved in transport or translational control.

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