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Identification of two proteins that bind to a pyrimidine-rich sequence in the 3′-untranslated region of GAP-43 mRNA

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ABSTRACT

GAP-43 is a membrane phosphoprotein that is important for the development and plasticity of neural connections. In undifferentiated PC12 pheochromocytoma cells, GAP-43 mRNA degrades rapidly ($t_{½}$ = 5 h), but becomes stable when cells are treated with nerve growth factor. To identify *trans*-acting factors that may influence mRNA stability, we combined column chromatography and gel mobility shift assays to isolate GAP-43 mRNA binding proteins from neonatal bovine brain tissue. This resulted in the isolation of two proteins that bind specifically and competitively to a pyrimidine-rich sequence in the 3′-untranslated region of GAP-43 mRNA. Partial amino acid sequencing revealed that one of the RNA binding proteins coincides with FBP (far upstream element binding protein), previously characterized as a protein that resembles hnRNP K and which binds to a single-stranded, pyrimidine-rich DNA sequence upstream of the c-*myc* gene to activate its expression. The other binding protein shares sequence homology with PTB, a polypyrimidine tract binding protein implicated in RNA splicing and regulation of translation initiation. The two proteins bind to a 26 nt pyrimidine-rich sequence lying 300 nt downstream of the end of the coding region, in an area shown by others to confer instability on a reporter mRNA in transient transfection assays. We therefore propose that FBP and the PTB-like protein may compete for binding at the same site to influence the stability of GAP-43 mRNA.

INTRODUCTION

The level of expression of a protein can be profoundly influenced by the stability of its mRNA. Half-lives of different mRNAs vary from several minutes to many hours and are generally thought to be determined by specific nucleotide sequences that may serve as binding sites for *trans*-acting factors (1–4). For example, mRNAs bearing multiple AU-rich elements are often unstable and when these domains are fused to an otherwise stable reporter mRNA, the hybrid mRNA shows decreased stability (5–10). Another example is the iron-responsive element (IRE) sequence found in the 3′-untranslated region (3′-UTR) of transferrin mRNA (11–13). In both of these cases, *trans*-acting proteins have been identified which bind to these sequences to mediate message stability (14–18).

The mRNA encoding one neuronal protein, GAP-43, decays rapidly in undifferentiated PC12 cells, but is stabilized when cells acquire a neuronal phenotype (19–22). GAP-43 is a membrane-bound phosphoprotein that has been linked to the development and plasticity of the nerve terminal (23–25). Expression of the protein can vary over a 100-fold range at different stages of neural differentiation, with high levels being synthesized during periods of axonal outgrowth and nerve terminal sprouting, but only very low levels in most mature neurons (23,24). This regulation involves changes at both the level of gene transcription (26,27) and mRNA stability (19–22). Several studies provide evidence that the 3′-UTR of GAP-43 mRNA includes elements that control stability. Fusion constructs containing this region inserted into a normally stable reporter mRNA become unstable in PC12 cells (28,29) and can be stabilized by treating cells with NGF (28). The importance of the 3′-UTR of GAP-43 mRNA is further suggested by its high degree of evolutionary conservation (26,29–32). Prior reports have described specific interactions between portions of the 3′-UTR of GAP-43 mRNA and particular brain proteins using band shift assays, UV cross-linking and northwestern blots (i.e. probing proteins immobilized on nitrocellulose membranes with radiolabeled RNA fragments) (29,33–35). The present studies utilized affinity chromatography and gel mobility shift assays to isolate GAP-43 mRNA binding proteins and to determine the specific binding sites involved. Here we identify two proteins that bind specifically and competitively to a pyrimidine-rich sequence that lies within a region in the 3′-UTR of GAP-43 mRNA shown to be a stability determinant (22).

MATERIALS AND METHODS

RNA synthesis

Plasmid pF1-1 (36), provided courtesy of Drs A.Rosenthal (Genentech, San Francisco, CA) and A.Routtenberg (Northwestern University), contains a 1.5 kb insert (see Fig. 1) encoding the 5′-UTR, coding sequence and most of the 3′-UTR of GAP-43 (protein F1) mRNA cloned into the *Eco*RI site of pGEM3 (Promega, Madison, WI). Two fragments of GAP-43 cDNA were

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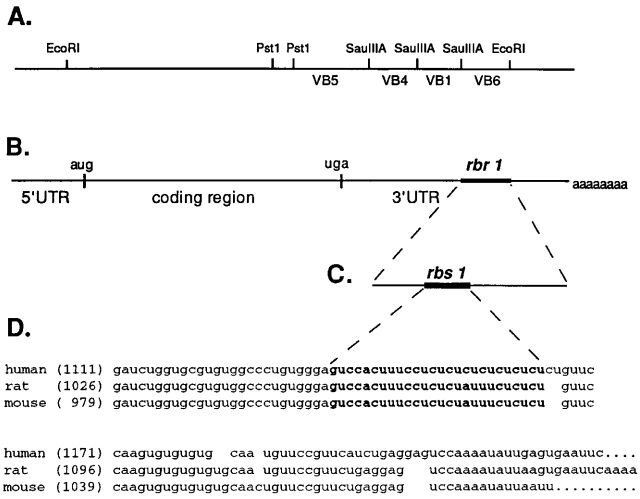

Figure 1. Structure of GAP-43 mRNA and evolutionary conservation of rbr1. (**A**) Restriction map of rat GAP-43 cDNA showing *Eco*RI, *Pst*I and *Sau*IIIA sites. Segments of the 3′-UTR present in plasmids pVB1, 4, 5 and 6 are indicated above. (**B**) Rat GAP-43 mRNA contains a 5′-untranslated region of 52–200 nt, a 630 base coding region and a 3′-UTR of ~600 nt (26,30–32). The 106 nt region that forms the U and M complexes is labeled rbr1. (**C**) rbr1 is expanded to show the location of rbs1, the 26 nt sequence that is sufficient for binding. (**D**) The sequence of rbs1, indicated in bold, is nearly identical in human, rat and mouse.

generated by digestion of pF1-1 with *Eco*RI and *Pst*I and were subcloned into pGEM3. *Sau*IIIA digestion of the *Pst*I–*Eco*RI fragment, encoding the 3′-half of the mRNA, produced four small fragments that we cloned into pGEM3 cut with a combination of *Eco*RI, *Pst*I and *Bam*HI. Plasmid pVB5 contains a fragment of 180 bases between the *Pst*I and first *Sau*IIIA sites, including the last 38 codons and the first 66 untranslated nucleotides; the fragment in plasmid pVB4 extends between the first and second *Sau*IIIA sites and is 114 nt in length; the fragment in pVB1 extends from the second *Sau*IIIA site to the third and is 116 nt in length; the fragment in pVB6, designated rbr1, extends from the third *Sau*IIIA site to the *Eco*RI site at the end of the clone. Plasmid pVB7 contains the rbs1 site, a 26 base fragment within rbr1 having the sequence TCCACTTTCCTCTCTATTTCTCTCTG, synthesized *in vitro* (Molecular Biology Facility, Children's Hospital) and cloned into pGEM3. Each plasmid was linearized with *Eco*RI and transcribed *in vitro* from the SP6 promoter in the presence of [α-^{32}P]UTP (DuPont/New England Nuclear, Boston, MA) as described in Sambrook *et al.* (37).

Mobility shift assays

Binding reactions employed methods similar to those of Leibold and Munro (13) and Konarska and Sharp (38). Each 10 µl reaction contained 4 µl of a protein sample, 5 µl 2× concentrated binding buffer [30 mM HEPES, pH 7.4, 20 mM KCl, 20% glycerol, 10 mM $MgCl_2$, 0.4 mM dithiothreitol, 0.2 mg/ml heparin and 1.2 U/µl RNasin (Promega)] and 1 µl (~10 ng) ^{32}P-labeled RNA. After incubation at room temperature for 15 min, RNase T1 (90 U) was added and the incubation continued for an additional 10 min at room temperature. The standard analysis involved separation of radiolabeled RNA fragments and protein–RNA complexes on non-denaturing RNase-free 0.25× TBE-buffered 4% polyacrylamide (30:1 acrylamide:bisacrylamide) gels pre-run at 25 V at 4°C for 30 min. After loading the binding reactions onto the running gel, electrophoresis continued at 15 V/cm for 1.5 h at 4°C. Protein–RNA complexes were visualized by autoradiography using Kodak XAR film at 4°C overnight. The specificity of protein–RNA complex formation was assessed by competition experiments, in which non-radioactive ribonucleotides were included in the binding reactions at a 10- or 100-fold excess. Competitors included the same nucleotide sequence, unlabeled, or a control sequence, in this case transcripts from bacteriophage λ. Additional competition experiments employed polyribonucleotides with lengths ranging from 300 to 3000 nt (Sigma) or synthetic deoxyribonucleotides with rbs1 sequences. In addition to controlling for the specificity of the binding reactions, these latter studies also helped establish whether oligonucleotides could serve as ligands in purification of GAP-43 mRNA binding proteins.

'Supershift' experiments were performed as described above except that 1 µl of an antibody generated against an internal peptide sequence of one of the binding proteins or control rabbit serum was incubated with the protein extract for 20 min at room temperature before the other components of the binding reaction were added. To generate this antibody, rabbits were injected with the peptide SVMTEEYKVPDGMVM, synthesized and cross-linked by Research Genetics (Huntsville, Alabama). This antibody specifically detected FBP on a western blot.

UV cross-linking of protein and RNA within the complexes

To visualize the RNA binding proteins, protein–RNA complexes were fractionated using non-denaturing gel electrophoresis and visualized by autoradiography as described above. Using the autoradiogram as a template, radiolabeled complexes were cut out from the gels and the components of the protein–RNA complexes were cross-linked by exposure of the gel to UV light (300 nm) at 4 cm from the light source (Stratalinker; Stratagene) for 7 min, equilibrated for 15 min in SDS sample buffer (39), heated to 95°C for 5 min and fractionated by PAGE (40). Gels were dried (Hoefer SE1160) and exposed to X-ray film to visualize the ^{32}P-labeled RNA–protein complexes.

Tissue and cell homogenates

Freshly dissected newborn calf brains, stripped of meninges and maintained at 4°C, were supplied by Pel-Freeze Inc. (Rogers, AR). The gray matter of the neocortex was dissected from the underlying white matter, maintaining the tissue continuously at 4°C (yield ~85 g/brain). Tissue was homogenized in a 4-fold excess (v/w) of a buffer containing 20 mM Tris–HCl, pH 7.4, 50 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 1 µg/ml leupeptin and 0.1 mM phenylmethylsulfonyl fluoride using a Waring blender (highest speed, 1 min). Homogenates were centrifuged in a Sorvall RC5C (SA 6000 rotor) at 17 000 *g* for 30 min. From 900 g wet weight bovine cortical gray matter, we obtained ~50 g soluble protein in 3.5 l buffer. In some experiments homogenates were obtained from cultured cells, rat brains or other tissues by homogenizing in the same buffer using a glass–Teflon homogenizer (0.1 mm gap, highest speed, 15 strokes, 4°C). Protein concentrations were determined using the Bradford assay (BioRad, Richmond, CA).

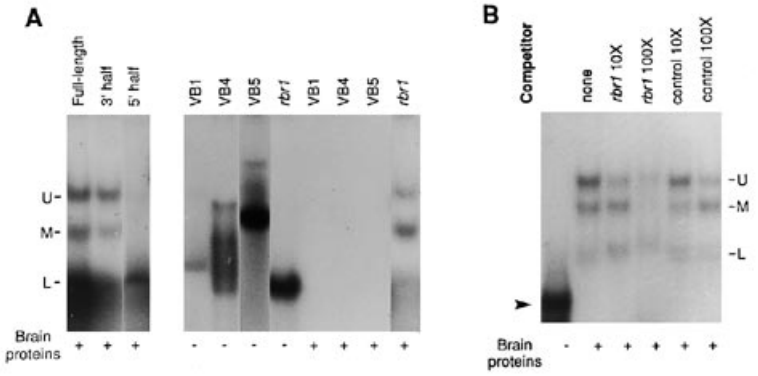

Figure 2. (**A**) rbr1, a region within the 3′-UTR of GAP-43 mRNA, forms three RNA–protein complexes. Portions of GAP-43 mRNA were transcribed from subclones of the *GAP-43* gene as described. In gel shift assays with cytosolic protein extracts from P4 rat brains, full-length GAP-43 mRNA, the 3′-half of the mRNA and the 106 nt rbr1 region all form the U, M and L complexes after T1 nuclease digestion. Under these conditions no complexes form with any of the other 3′ fragments, VB1, VB4 or VB5. A complex similar in size to L also forms with the 5′-end of the mRNA. The four middle lanes show migration of the probes without T1 digestion and without protein addition. (**B**) Specificity of protein–RNA interactions. Lane 1, migration of ^{32}P-labeled rbr1 alone (arrow); lane 2, retardation of rbr1 after incubation with 100 µg neonatal rat brain cytosolic proteins (protein–RNA complexes are labeled U, M and L); lanes 3 and 4, at a 100-fold molar excess, non-radioactive rbr1 competes with the labeled probe for formation of the U and M complexes; lanes 5 and 6, control RNA fragments derived by transcribing fragments of λ phage DNA fail to compete with radiolabeled rbr1.

Protein purification

To purify sufficient quantities of the RNA binding proteins for sequencing, we used a series of columns based upon charge, heparin binding and affinity for oligonucleotide sequences. The pH of the soluble bovine brain protein fraction was adjusted to 8.0 with NaOH and the sample was applied in two batches to a column of pre-equilibrated diethylaminoethyl cellulose (DE-52; Whatman, Maidstone, UK), 5 × 25 cm, at a flow rate of 3 ml/min. The proteins were eluted sequentially with 5 column vol. each of buffer A alone (20 mM Tris–HCl, pH 8.0, 50 mM KCl, 5 mM EGTA and 1 mg/ml leupeptin), then buffer A containing 200 mM KCl and 1 M KCl. The column was washed with 20% ethanol and re-equilibrated between runs. Gel shift assays revealed that the proteins of interest remained in the flow-through fraction. This fraction (containing ~12 g protein/3.5 l) was adjusted to pH 7.4 and applied to a heparin–Sepharose (Sigma) column (2.5 × 20 cm) at 2 ml/min, 4°C. The column was washed sequentially with 3 vol. buffer B (20 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 0.1 mM dithiothreitol and 10% glycerol) containing 50 mM NaCl and eluted with 5 vol. each of buffer B containing 100, 200, 300, 400, 500 and 1000 mM NaCl. Fractions eluting with 200 and 300 mM NaCl were found in band shift assays to contain the RNA binding proteins. These fractions were treated with 10 U/ml recombinant RNasin (Promega) and applied directly to the oligonucleotide column at a flow rate of 1 ml/min without changing the salt concentration, as preliminary studies had indicated that the RNA binding proteins remain bound to the final column in the presence of 300 mM NaCl. The oligonucleotide column contained 1.5 mg poly(U) covalently bound to Sepharose (Sigma) and was pre-equilibrated with buffer B treated with 10 U/ml recombinant RNase inhibitor (RNasin; Promega). Following application of the sample, the column was washed with 5 vol. buffer B containing 200 µg/ml tRNA, 1 mg/ml heparin and 300 mM NaCl, then eluted with the same buffer containing 500, 1000 and 2000 mM NaCl. Fractions from all stages of the purification were analyzed by band shift assays and by SDS–PAGE, with specific conditions indicated in the figures.

Fractions enriched in the RNA binding proteins were dialyzed against buffer B (without glycerol) and then concentrated to 100 µl using a Centricon 10 microconcentrator (Amicon, Bedford, MA). Samples were separated on SDS–polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes (41). Membranes were stained with 1% Ponceau S and destained as described (42). Protein bands were cut out, washed three times in HPLC grade water and digested with trypsin. Peptides were separated by reversed phase HPLC and selected peaks were tested for purity and size by mass spectroscopy. Sequencing was carried out by the Harvard Microchemistry Facility.

RESULTS

Identification of binding domains in GAP-43 mRNA

^{32}P-Labeled RNA was transcribed from the entire GAP-43 cDNA in plasmid pF1-1 (Fig. 1A) and used in band shift assays. Three protein–RNA complexes (U, M and L) formed when cytoplasmic proteins of the neonatal bovine brain were incubated with the full-length RNA (Fig. 2A, lane 1). In this and all other mobility shift assays, the reaction mixture was treated with RNase T1 prior to electrophoresis (13,38), resulting in a complex whose size reflects the protein plus a bound RNA fragment. The U, M and L complexes appeared when the 3′-half of GAP-43 mRNA alone was used in the assay (lane 2), whereas the 5′-half of the mRNA formed only one complex which migrated similarly to L (lane 3). To define further the region to which proteins bind, four non-overlapping fragments (VB5, VB4, VB1 and VB6, Fig. 1A), representing most of the 3′-half of GAP-43 mRNA, were transcribed *in vitro* in the presence or absence of [^{32}P]UTP and used in mobility shift assays. Only the region designated as RNA binding region 1 (rbr1), a 106 nt sequence that begins 300 nt downstream of the termination codon, formed the three complexes (last lane). Figure 1B and D show the position and sequence of rbr1 within GAP-43 mRNA.

Competition assays were carried out to evaluate the specificity of the protein–RNA interactions. Figure 2B shows that when a

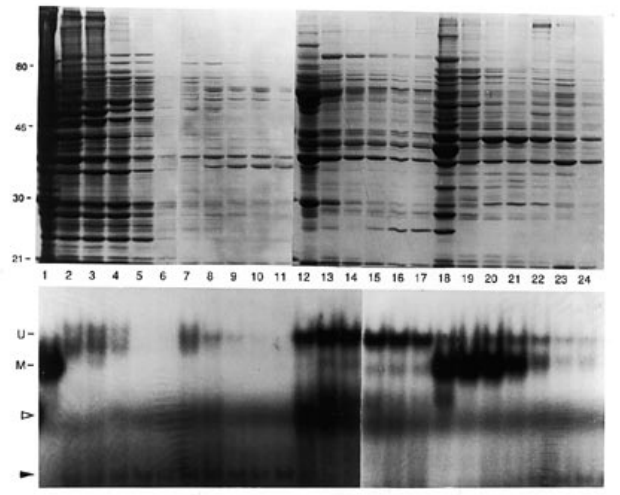

Figure 3. Heparin affinity chromatography. Unbound proteins from the DEAE column were applied to a heparin–Sepharose column (pH 7.4) and eluted with increasing salt concentrations. Protein composition was visualized by SDS–PAGE (top), while binding activity was assayed by gel retardation (bottom). Lane 1, starting material (50 µl from 3.5 l); lane 2, unbound proteins from the heparin column (50 µl from 3.5 l); lanes 3–6, column washes: fractions eluted with 100 mM (fractions 7–11), 200 mM (lanes 12–17), 300 mM (lanes 18–23) and 500 mM NaCl (lane 24). Proteins that form the U and M proteins eluted at 200 and 300 mM NaCl respectively (lanes 3–24 contain 50 µl from 50 ml and represent a 70-fold higher proportion of the fractions than in lanes 1 and 2). U-forming activity is not seen in lane 1, since levels of the RNA probe were limiting and the M complex forms preferentially, as described in the text. The open arrow indicates the position of the undigested rbr1 probe alone. The 26 nt fragment generated by RNase T1 digestion of rbr1 is shown by the closed arrow.

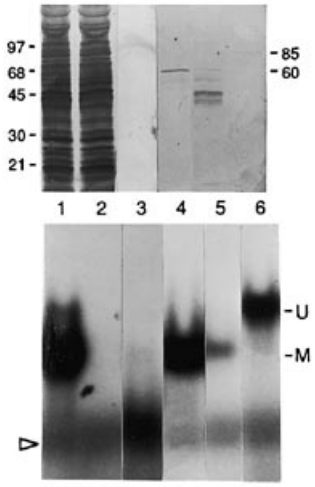

Figure 4. Poly(U) affinity chromatography. Heparin column fractions containing the proteins of interest were applied to a poly(U) column (pH 7.4) and eluted with increasing concentrations of NaCl (3 ml/fraction). Fractions were analyzed by SDS–PAGE (top; 2 µl samples separated on a minigel and silver stained) and band shift assays (bottom). Lane 1, starting material; lane 2, unbound fraction; lanes 3, material eluted with 200 mM NaCl; lane 4, 500 mM NaCl; lane 5, 1000 mM NaCl; lane 6, 2000 mM NaCl. Positions of the 60 and 85 kDa RNA binding proteins are indicated on the top right; U, M, upper and middle protein–RNA complexes. Arrowhead, unbound probe.

100-fold molar excess of non-radioactive rbr1 RNA was added at the beginning of the incubation, it competed with the radiolabeled probe, thus reducing the level of radioactivity in the U and M complexes. There was only a slight reduction in labeling of the L complex. This result is significant because control RNA sequences, transcribed from fragments of bacteriophage λ, failed to fully displace radioactive rbr1 from any of the three complexes when present at a 100-fold excess. These data suggest that the U and M complexes represent specific interactions between nucleotide sequences in fragment rbr1 and particular brain proteins.

Isolation of the proteins that form the U and M complexes

After separating the cytosolic proteins from newborn calf gray matter by DEAE–cellulose column chromotography (pH 8.0), the proteins that form the M and U complexes remained in the flow-through fraction. When this fraction was applied to a heparin–Sepharose column, the proteins that form the U and M complexes bound and were eluted with 200 mM and 300 mM NaCl respectively (Fig. 3). Fractions containing these proteins were applied to a polyuridylic acid [poly(U)]–Sepharose column. The proteins that form the M complex bound to this column with high affinity and eluted at 500 mM NaCl (Fig. 4). A comparison of the band shift assays with the protein staining patterns indicated that formation of the M complex correlated well with the presence of a prominent protein migrating at 60 kDa. The major protein derived from fractions that formed the U complex eluted at 2000 mM NaCl and had an apparent molecular weight of 85 kDa.

Peptide sequencing and identification

Two peptides from the 60 kDa protein were found by microsequencing to have an amino acid sequence highly similar to an identified pyrimidine tract binding (PTB) protein of rat and human (43–45; Fig. 5). In the M1 peptide, 10 of 12 amino acids are identical to the human and rat PTB sequence. In the M2 peptide, 18 of the 30 amino acids are identical to the human and rat PTB sequences. The sequences of three peptides (U1, U2 and U3) from the 80 kDa protein found in fractions that form the U complex are nearly identical with amino acid sequences from far upstream element binding protein (FBP) (Fig. 5). FBP is a protein that regulates *myc* expression by binding to a pyrimidine-rich, single-stranded far upstream sequence element (FUSE) (46). To confirm that the protein in the U complex is in fact FBP, we used a polyclonal antiserum directed against an FBP peptide in a gel mobility shift assay (47). On a Western blot containing cytosolic proteins of the bovine brain, the antibody reacted specifically with a protein of 85 kDa (data not shown), the same size as the purified protein. When this antiserum, but not when control antiserum, was added to P4 brain cytosol prior to the binding reaction, migration of the U complex was electrophoretically retarded compared with the U complex itself (Fig. 6).

Confirmation of complex composition by UV cross-linking

Separation of UV cross-linked radiolabeled protein–RNA complexes on SDS–polyacrylamide gels provided additional information on the molecular sizes of the proteins that form the M and U complexes. After protein–RNA complexes were allowed to form in solution, samples were treated with RNase T1 to remove RNA not bound to protein, then fractionated using non-denaturing PAGE. The RNA–protein complexes were visualized by autoradio-

Protein from the M complex

PTB 123-134 GQPIYIQ*F*SNHK
M1 *N*QPIYIQ*Y*SNHK

PTB 298-325 *GLSVPNVH*GALAPLAIP*S*AAAAAAAAGR
M2 *ETSLLAVP*GAL*S*PLAIP*N*AAAAAAAAGR

Protein from the U complex

FBP 99-118 SVMTEEYKVPDGMV*G*FIIGR
U1 SVMTEEYKVPDGMV*M*FIIGR

FBP 426-430 LFTIR
U2 LFTIR

FBP 593-626 MGQAV*VP*APTGAPPGGQPDYSAAW EYYRQQAAYY
U3 MGQAV *DA*PTGAPPGGQPDYSAAW*P*EYYRQQAAYY

Figure 5. Peptides from the two RNA binding proteins resemble portions of two previously identified polypeptides. (**Top**) Two peptides from the protein that forms the M complex were found by microsequencing to have an amino acid sequence highly similar to an identified pyrimidine tract binding (PTB) protein of rat and human (43–45). In the first sequence, 10 out of 12 nucleotides are the same, while in the second, 18 of the 30 amino acids are identical to the human and rat PTB sequences. These results suggest that the protein that participates in the M complex is in the PTB family of proteins. (**Bottom**) The sequences of three peptides from the protein that is part of the U complex are nearly identical with amino acid sequences from the single-stranded DNA binding protein FBP, which regulates *myc* expression by binding to a FUSE (46). Amino acids that differ between the sequences obtained in these studies and the identified proteins are shown in italic.

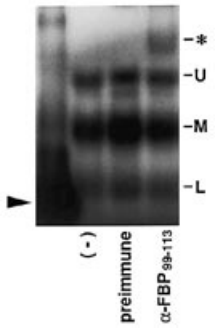

Figure 6. Antiserum raised against an FBP peptide supershifts the upper band. The probe alone is designated by an arrow. In the last three lanes, rat brain cytosolic proteins have been mixed with radiolabeled rbr1 alone, with preimmune sera or with antibodies raised against the FBP peptide. In the fourth lane, the supershifted band containing rbr1, FBP and anti-FBP antibodies is marked by an asterisk.

graphy, excised from the gel and the components of these complexes were covalently cross-linked by exposure to UV light at 300 nm. Radiolabeled complexes were denatured and fractionated by SDS–PAGE. The major protein with covalently bound ^{32}P-labeled RNA from the M complex migrated with an apparent molecular size of 67 kDa, while the one from the U complex migrated at 97 kDa (Fig. 7). Since the RNA fragment linked to these proteins is 26 nt, its size would be ~7.5 kDa. Thus, the predicted molecular weights of the two proteins alone are ~60 and 90 kDa, in good agreement with the sizes of the proteins we have isolated.

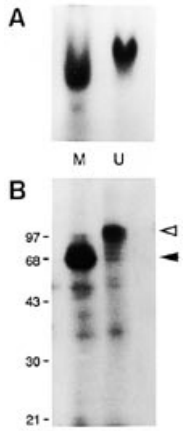

Figure 7. UV cross-linking of protein and RNA within the complexes. (**A**) The M and U complexes, composed of ^{32}P-labeled rbr1 and the associated binding proteins are visualized in band shift assays. (**B**) Labeled bands were excised from non-denaturing gels and the protein–RNA complexes were cross-linked by exposure to UV, separated on SDS–polyacrylamide gels and visualized by autoradiography. The labeled RNA–protein complexes migrate with apparent molecular weights of 97 (open arrow) and 67 kDa (closed arrow).

Specificity of protein–RNA interactions

Analysis of the nucleotide sequence of rbr1 indicates that RNase T1, which was routinely added after the binding reactions were completed, would digest rbr1 into one large 26 nt fragment along with many small fragments of 2–10 nt. In RNase T1-treated samples containing no added RNA binding proteins, an RNA fragment of this length appeared near the bottom of the gel (closed arrows in Fig. 3), suggesting that the 26 nt fragment is normally part of the complex. When this 26 nt RNA was synthesized *in vitro* from pVB7 and incubated with the protein extract, this RNA sequence alone was able to form the U, M and L complexes (Fig. 8A). The 26 nt RNA is designated RNA binding site 1 (rbs1) and is highlighted in Figure 1. This fragment contains both C and U, suggesting that both pyrimidines contribute to binding. Competition experiments show that at 100× poly(U) but not poly(A), poly(C) nor poly(G) succeeded in competing for formation of the U and M complexes (data not shown). Further competition experiments demonstrate that poly(CU), a random polymer of C and U many kilobases in length, fully competed for rbr1 binding when present at 10× the concentration of the labeled probe, whereas poly(U), a mixture of similarly sized homopolymers, required a higher concentration to compete (Fig. 8B). It should be noted that the polynucleotides may contain multiple binding sites, whereas rbr1 probably contains only one. Two deoxynucleotide sequences, one encoding the 5′ 14 nt of rbs1 and the other the 3′ 12 nt, were tested for their ability to compete with the binding of rbr1 when present at 100× molar excess. The 5′ sense fragment completely displaced rbr1 from both the U and M complexes, whereas the 3′ sense fragment showed only partial competition for the upper complex (data not shown).

DISCUSSION

This study combined column chromatography and gel shift assays, using restricted portions of GAP-43 mRNA, to identify

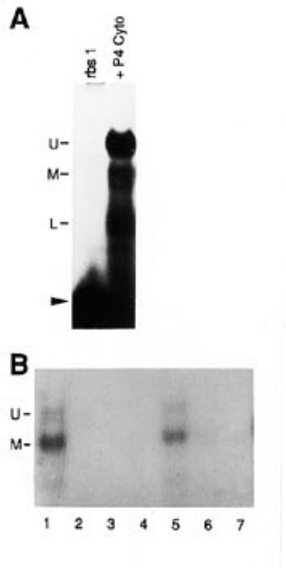

Figure 8. (**A**) A 26mer RNA transcript (first lane) corresponding to rbs1 forms the U, M and L complexes (second lane). (**B**) A polynucleotide consisting of random cytidine–uridine sequences competes for the binding proteins more effectively than does poly(U). Lane 1, complexes formed when rbr1 RNA was incubated with P4 rat brain cytosol fraction; lanes 2–4, poly(CU) at either 10× (lane 2), 100× (lane 3) or 1000× (lane 4) molar excess competed for the binding proteins; lanes 5–7, poly(U) failed to displace the binding proteins when present at 10× molar excess (lane 5), but competed when present at either a 100× or 1000× excess (lanes 6 and 7).

cis- and *trans*-acting factors that may contribute to the post-transcriptional regulation of GAP-43 expression. We identified a 26 nt region within the 3′-UTR to which two proteins bind specifically and competitively. This region lies within a sequence shown by others to confer instability upon GAP-43 mRNA or a reporter mRNA (22,28). The two binding proteins were isolated to homogeneity and subjected to microsequencing. The protein that forms the larger protein–RNA complex (U) was found to coincide with the FUSE binding protein (FBP), initially isolated by virtue of its affinity to a single-stranded nucleotide sequence far upstream from the promoter region of c-*myc* (46). FBP shares significant sequence homology with the RNA binding protein hnRNP K. Consistent with these properties, we find that FBP binds to both a 26 nt region in the 3′-UTR of GAP-43 mRNA and to the corresponding single-stranded deoxyribonucleotide sequence. Further evidence that FBP is the protein that forms the U complex comes from ‘supershift’ assays, in which an antibody generated to a peptide sequence within FBP was found to bind to the complex and alter its electrophoretic migration. A second protein which forms the M complex at the same binding site was shown by partial amino acid sequencing to resemble PTB, an identified polypyrimidine tract binding protein (44,45). Several variants of PTB have been identified and it is likely that the protein that forms the M complex represents a member of this family (45).

The binding region, termed rbr1, lies 300 nt downstream of the translation stop codon. The specificity of binding to this region was demonstrated by the fact that an excess of non-radioactive rbr1 competed effectively with the radioactive rbr1 probe for formation of the complexes, whereas several other mRNA sequences failed to compete. Within rbr1, the binding region was narrowed down further to a 26 nt pyrimidine-rich sequence, rbs1, which appears to be both necessary and sufficient for formation of the U and M complexes. Consistent with the high content of U and C in this sequence, the binding of the proteins that form the U and M complexes could be displaced by excess poly(U) and even more successfully by a polyribonucleotide consisting of random cytidine–uridine sequences. The position of the rbs1 sequence within the 3′-UTR of GAP-43 mRNA is indicated by an asterisk in Figure 9, which depicts the most stable conformation of the 3′-UTR predicted by the Genetics Computer Group program MFOLD (48,49). rbs1 lies within an exposed loop and is likely to be accessible for protein binding. Across species, the stem–loop structure that includes rbs1 has been highly conserved (26,29–32).

Since FBP and the PTB-like protein bind to this same region, competition between the two would be expected and was in fact observed during protein purification. Whereas the starting material from bovine brain homogenate formed the M complex almost exclusively in experiments where the RNA probe was limiting, further separation yielded fractions that also formed the U complex as the concentration of the other protein declined (Figs 3 and 4). Binding studies using varying concentrations of purified protein fractions have demonstrated this competition explicitly (V.Baekelandt, L.I.Benowitz, F.Vandesande and N.Irwin, unpublished data).

In the initial gel shift assays, three protein–RNA complexes were visualized when cytoplasmic proteins from the neonatal rat or bovine brain were incubated with rbr1. Whereas the binding of FBP and the PTB-like protein to radioactive rbr1 could be competed with an excess of non-radioactive rbr1 but not by an unrelated sequence, the protein that formed the third complex (L) did not meet these criteria and was therefore not pursued further. In the final stage of purification, FBP and the PTB-like protein were among a very small group of polypeptides that showed high affinity binding to poly(U). The most prominent band in the fractions which formed the M complex had a molecular size of 60 kDa, which coincides with the size predicted from the UV cross-linking experiment. Likewise, the latter experiments indicated a size of 90 kDa for the protein that forms the U complex. This is slightly larger than the size of the protein visualized by SDS–PAGE from column fractions that formed the U complex. Using similar UV cross-linking methods, Kohn *et al.* (29) recently reported that three proteins with apparent molecular weights of 85, 60 and 40 kDa were capable of binding to the 3′-UTR of GAP-43 mRNA and that the binding region was likely to be a polypyrimidine stretch, since poly(U) competed for binding. Based upon these properties, it would appear that the 85 and 60 kDa binding activities described by those authors coincide with the two proteins we have isolated here. In addition, Chicurel *et al.* (35) reported that the microtubule-associated protein MAP1 binds to a region in the 3′-UTR of GAP-43 mRNA upstream of rbs1.

It is noteworthy that the proteins identified in this study, or closely related family members, have other functions, including transcriptional regulation (in the case of FBP; 46), RNA splicing and translational control (in the case of PTB; 43–45). Likewise, proteins that bind to the iron-responsive element motif have more than one role: they regulate both the stability of mRNA encoding the transferrin receptor and translation of ferritin mRNA (11–13). Perhaps many single-stranded nucleotide binding proteins may prove to be multifunctional.

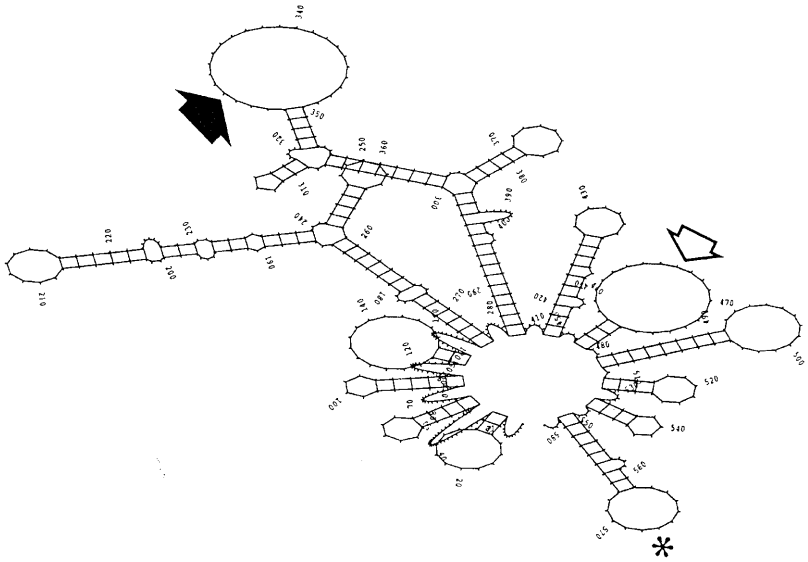

Figure 9. MFOLD structure of the 3′-UTR of GAP-43 mRNA. In its most stable conformation (49,50), rbs1 (black arrow) lies in a single-stranded loop accessible to potential binding proteins. The poly(A) addition signal (asterisk) and an AUUUA sequence (open arrow) are also on exposed loops, accessible to the poly(A) binding protein (54) and AUBF (18) respectively.

Although rbs1 is one important determinant of GAP-43 mRNA stability, regulation of this stability by NGF requires regions 5′ of rbs1 (22,28). In conformity with this, formation of the M and U complexes in PC12 cell extracts was found here to occur irrespective of whether cells were treated with NGF. Nevertheless, it remains possible that binding of the PTB-like protein and/or FBP to rbs1 is important for determining stability of the mRNA in other instances, in which stability is mediated by regulatory signals other than NGF, or that these proteins interact with other proteins not detected here that bind to the NGF-responsive element of the mRNA. Preliminary *in vitro* experiments indicate that FBP facilitates mRNA degradation, while the PTB-related protein, which has a higher affinity for rbs1 than FBP, inhibits the effect of FBP on mRNA degradation. Thus, FBP binding may contribute to destabilizing GAP-43 mRNA, whereas the PTB-related protein might act competitively to render the mRNA more stable. In addition to binding to GAP-43 mRNA, it is possible that the PTB-related protein and FBP could bind to sites within other brain mRNAs. Pyrimidine-rich sequences have been implicated in the stability of the mRNAs encoding tyrosine hydroxylase and the amyloid precursor protein (50–52) and it would be of considerable interest to determine whether the same proteins identified here also play a role in regulating the stability of other mRNAs during neural differentiation.

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