Regulation of mRNA turnover is a critical control mechanism of gene expression and is influenced by ribonucleoprotein (RNP) complexes that form on cis elements. All mRNAs have an intrinsic half-life and in many cases these half-lives can be altered by a variety of stimuli that are manifested through the formation or disruption of an RNP structure. The stability of α-globin mRNA is determined by elements in the 3′ untranslated region that are bound by an RNP complex (α-complex) which appears to control the erythroid-specific accumulation of α-globin mRNA. The α-complex could consist of up to six distinct proteins or protein families. One of these families is a prominent polycytidylate binding activity which consists of two highly homologous proteins, α-complex proteins 1 and 2 (αCP1 and αCP2). This article focuses on various methodologies for the detection and manipulation of αCP1 and αCP2 binding to RNA and details means of isolating and characterizing mRNA bound by these proteins to study mRNA turnover and its regulation.

The processing and ultimately the turnover of mRNA are complex processes that are highly regulated. Within a cell, RNA is not usually in a naked form, rather it is bound by various RNA-binding proteins forming ribonucleoprotein (RNP) complexes that influence processing and turnover events (1). Therefore, it is critical to isolate and characterize the various RNA-binding proteins as a way of delineating the mechanism of posttranscriptional events. A major emphasis has been placed on the isolation of proteins involved in mRNA processing, yet relatively little is known pertaining to the trans factors involved in mRNA turnover. Identification of RNA-binding proteins and characterization of the binding properties of proteins that influence mRNA turnover will facilitate the mechanistic understanding of this process and provide avenues to regulate mRNA stability.

The globin mRNAs, which are among the most stable mRNAs, with estimated half-lives of as long as 60 h (2, 3), provide an ideal model system to study determinants of mRNA stability. The stability of human α-globin mRNA is conferred by a pyrimidine-rich region in the 3′ untranslated region (3′UTR) that forms a specific RNP complex (α-complex) (4–6). The formation of the α-complex is sensitive to polycytidylate [poly(C)] competition, suggesting the presence of a poly(C) binding activity within this complex.

Mammalian cells contain two major classes of poly(C)-binding proteins, the first being the hnRNP K protein (68 kDa) (7) and the second being the 43-kDa poly(C) binding activity within the α-complex, α-complex proteins 1 and 2 (αCP1 and αCP2) (8) [also referred to as PCBP1 and PCBP2, respectively, in (9)]. The hnRNP K protein is one of approximately 20 abundant pre-mRNA-binding proteins that are predominately nuclear but many of which (including hnRNP K) shuttle between the nucleus and cytoplasm and are involved in various aspects of mRNA maturation (1, 10). Cloning of hnRNP K enabled identification of a new RNA-binding domain, the K homology (KH) domain (11).

αCP1 and αCP2 are highly homologous (>90% similar) proteins encoded by two distinct genes mapping to chromosomes 2p12-13 and 12q13.12-q13.13, respectively (12). Both proteins contain three KH
domains each. αCP2 was originally reported by Hahm et al. (13) as a protein that copurified with a lymphocyte transcription factor. The presence of KH domains in αCP2 led the authors to speculate it might be an hnRNP protein and termed it hnRNP X. A similar murine isoform was identified by Goller et al. (14) as an oligo(C)-binding protein and was termed mouse C-binding protein, mCBP, based on its binding activity. Human αCP1 was initially reported by Aasheim et al. (15) as clone sub2.3, which had similarity to αCP2 and also contained poly(C) binding activity. Leffers et al. (9) isolated human αCP2 [referred to as poly(C)-binding protein 2, PCBP2, based on its binding activity]. A functional role for αCP1 and αCP2 was first reported by Kiledjian et al. (8). The poly(C) binding activity within the α-globin mRNA-stabilizing α-complex was purified and shown to be the αCP1 and αCP2 proteins. This finding therefore implicated these proteins in mRNA stability. αCP1 and αCP2 proteins appear to be ubiquitous in humans (9, 15) and their presence in nonerythroid cells suggests their involvement in the stability of nonglobin mRNA as well. More recently, both αCP1 and αCP2 have been implicated in poliovirus replication and translation (16–18), while αCP1 has also been implicated in the translational regulation of the 15-lipoxygenase mRNA (19).

The αCP1 and αCP2 proteins are members of a growing family of RNA-binding proteins that contain a KH domain. The KH domain is an evolutionarily conserved RNA binding motif, originally identified by Siomi et al. (11) as a sequence repeated three times in the hnRNP K protein, and is similar to other known RNA-binding proteins. The KH domain is approximately 60 amino acids long and has a βαβαββα structure with three antiparallel β sheets on one surface positioned against three α helices on another (20). An invariant Gly-X-X-Gly sequence, where the X is usually a positively charged amino acid, is contained within the loop between helixes 1 and 2 and is thought to be essential for RNA binding (21). The first and third KH domains of both αCP1 and αCP2 appear to be essential for poly(C) binding, while the contribution of the second KH domain is currently unclear (22).

This report summarizes methods pertaining to the detection of αCP binding to RNA. It describes the purification of αCP1 and αCP2 from erythroid cells as well as the purification of recombinant bacterially expressed protein. It details methods to detect the RNA binding properties of αCP1 and αCP2, in addition to procedures to isolate RNA bound by these proteins, and describes the isolation and resolution of an RNP complex containing these proteins.

## DESCRIPTION OF METHODS

### Purification of the αCP1 and αCP2 Proteins

Methods are presented below that describe the purification of endogenous αCP1 and αCP2 from eukaryotic cell extract or recombinant bacterially expressed proteins. The endogenous protein provides the advantage of isolating naturally modified form(s) of the proteins, while the recombinant protein provides the convenience of a quick purification as well as ample quantities. However, it should be noted that endogenous αCP1 and αCP2 copurify and do not resolve when isolated from eukaryotic cells using the procedure described below. Recombinant protein must be used whenever purified αCP1 or αCP2 only are required. Although αCP1 and αCP2 are present in both the nuclear and cytoplasmic fractions, cytoplasmic S100 extract is a better source for these proteins because it minimizes copurification of nuclear hnRNP proteins. The abundant nature of these proteins in the S100 extract allows for a convenient and easy purification.

#### Isolation of S100 and S130 Extracts

All steps were performed with ice-cold solutions at 4°C using approximately $10^{10}$ human erythroleukemia K562 cells. Cells were collected by a 3-min centrifugation at 400g and washed twice with phosphate-buffered saline (PBS). (The cell pellet could at this point be stored at $-70°C$ or lysed to isolate extract.) Cells were lysed with 25 strokes of a type B pestle in a Dounce homogenizer in a hypotonic lysis buffer ($2 \times 10^8$ cells/ml lysis buffer) containing 20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM MgCl$_2$, 0.5 mM dithiothreitol (DTT), 2 μg/ml leupeptin, and 0.5% aprotenin. Cell debris and nuclei were pelleted with a 5000g spin for 5 min and the KCl concentration was adjusted to 40 mM by the addition of 2 M KCl. The supernatant was centrifuged at 100,000g for 1 h (28,000 rpm in a Beckman SW40 rotor). The thin layer of lipid at the top of the supernatant was aspirated and the remaining S100 supernatant was collected and glycerol added to 5% (v/v). The resulting extract [usually 5–10 mg/ml as determined by a Bio-Rad Protein Assay reagent with bovine serum albumin (BSA) standards] was either
used directly for purifications or aliquoted and stored at −70°C.

Isolation of the cytoplasmic S130 extract was adopted from Brewer and Ross (23) and is essentially similar to the S100 extract with the following modifications: (1) the lysis buffer used consisted of 10 mM Tris–HCl, pH 7.5, 1 mM potassium acetate, 1.5 mM magnesium acetate, and 2 mM DTT; (2) following lysis with a Dounce homogenizer, the nuclei were removed with a 12,000g centrifugation and the supernatant was layered over the above lysis buffer containing 30% (w/v) sucrose. The sample was then centrifuged at 130,000g for 2.5 h (31,000 rpm in a Beckman SW40 rotor) and the S130 supernatant was collected without disturbing the S130–sucrose interface. Glycerol was added to a final concentration of 5% (v/v) and the extract frozen in aliquots at −70°C.

Purification of Cellular αCP1 and αCP2 Proteins

S100 extract isolated from approximately 10^{10} cells was used as the starting material for the purification scheme. Endogenous RNP complexes were dissociated by treatment with 400 U/ml micrococcal nuclease (Pharmacia) in the presence of 1 mM CaCl_{2} at 30°C for 20 min and the reaction was stopped with the addition of 5 mM EGTA. The small fraction of proteins (presumably RNA-binding proteins) that precipitated out of solution once the RNA was degraded was removed by a centrifugation at 10,000g for 10 min. The cleared supernatant was loaded onto a 30-ml SP-Sepharose (Pharmacia) column equilibrated in HEG-40 (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 40 mM KCl) at a flow rate of 1 ml/min on a Pharmacia HPLC column. The column flow-through was directly loaded onto a 30-ml DEAE-Sephaloc (Pharmacia) column also equilibrated in HEG-40 at the same flow rate. The KCl concentration of the DEAE-Sephaloc flow-through was increased to 300 mM and was then bound to a single-stranded (ss) DNA cellulose affinity column (5-ml column; USB), at 0.75 ml/min in HEG-300. Proteins interacting nonspecifically with the ssDNA were washed with 3 column vol of HEG-300 containing 1 mg/ml heparin. Bound protein was eluted with a linear KCl gradient from 300 mM to 1.5 M over 20 min at a flow rate of 1 ml/min. Half-milliliter fractions were collected and 20 μl of each aliquot was resolved by 12.5% SDS–PAGE. The presence of the protein was detected by either a Northwestern or Western assay and visualized by silver staining (8).

Most of the αCP1 and αCP2 proteins eluted at approximately 800 mM KCl.

Purification of Recombinant αCP1 and αCP2 Proteins

Whenever recombinant proteins are required, the αCP1 and αCP2 proteins were expressed as glutathione S-transferase (GST) fusion proteins and purified using glutathione-Sepharose beads according to the manufacturer (Pharmacia). Full-length fusion protein was isolated away from proteolytic products with the use of ssDNA cellulose affinity chromatography as follows. The glutathione-Sepharose column eluate was adjusted to 100 mM KCl in HEG (HEG-100) and bound to a ssDNA column as described previously. Protein was eluted with a 0.1 to 1 M KCl gradient over 20 min and fractions were collected and tested as described above. The majority of the poly(C)-binding competent protein eluted at approximately 400 mM KCl.

We were unable to use the traditional pGEX system to separate the GST domain from αCP1 and αCP2 using thrombin protease digestion because the protein was degrading. However, efficient isolation of bacterially expressed αCP1 and αCP2 was obtained using the pGEX-6p system (Pharmacia) in which the GST domain can be cleaved from the fusion protein using a recombinant human rhinovirus type 14 3C protease (PreScission protease, Pharmacia). The high specificity of the protease to the primary, as well as the higher-order structure of the recognition site, minimizes aberrant proteolysis elsewhere in the fusion protein. As shown in Fig. 1, recombinant GST–αCP1 fusion protein (~70 kDa) was overexpressed in Escherichia coli BL21 cells on induction with 0.2 mM isopropyl-β-D-thiogalactoside (IPTG) for 2 h (compare lanes 1 and 2). The fusion protein was purified using glutathione-Sepharose resin (lane 3) according to the manufacturer (Pharmacia). The beads were washed five times in PBS containing 0.5% Triton X-100 and once in cleavage buffer (50 mM Tris–HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) prior to incubation with 2 U protease overnight at 4°C on a nutator. Following cleavage, the supernatant containing cleaved αCP1 was collected (lane 4). The protease that contains a GST domain and the GST domain of the original GST–αCP1 fusion protein were both retained on the glutathione column and were not released into the supernatant. Since the purified fusion protein preparation also contains proteolytic products that retain the GST domain, cleavage also releases trun-
cated αCP1 protein (lane 4). Separation of the full-length αCP1 from the proteolytic products could be achieved by purification on a ssDNA column as described above.

Testing αCP1 and αCP2 Binding to RNA
Ribohomopolymer Binding Assays

The first biochemical activity attributed to αCP1 and αCP2 was their poly(C) binding activity. One approach to determining if a protein is competent to bind ribonucleic acids is to use ribohomopolymers coupled to agarose beads. αCP1 and αCP2 were transcribed and translated in vitro in the presence of [35S]methionine (Amersham) using the TNT rabbit reticulocyte system (Promega Biotech) as per the manufacturer. Approximately 10⁵ trichloroacetic acid-precipitable cpm of in vitro-produced protein was bound to 30 μg of the ribonucleotide homopolymer–agarose beads (Sigma) in HB buffer (10 mM Tris–HCl, 250 mM NaCl, 1.5 mM MgCl₂, 0.5% v/v Triton X-100, 2 μg/ml each of leupeptin and pepstatin, and 0.5% v/v aprotinin) for 15 min at 4°C. Protein bound to the ribonucleotide homopolymer–agarose beads was pelleted with a 3-s spin in the microfuge and nonspecifically interacting proteins were washed off with HB buffer containing 500 mM NaCl and 1 mg/ml heparin for an additional 10 min. The beads, along with the bound protein, were pelleted with a brief spin in the microfuge, the supernatant was aspirated, and the beads were rinsed with 1 ml of HB buffer. The washes were repeated four more times and followed by an additional rinse in HB buffer without Triton X-100. Excess solution was removed with a gel loading tip. The dried beads were resuspended in 30 μl SDS–PAGE loading buffer and boiled for 3 min to elute bound protein from the nucleic acid. The eluted protein was resolved on a 12.5% SDS–PAGE gel with 0.75-mm spacers. The use of a thinner gel minimizes quenching of the 35S and allows direct exposure of the dried gel onto X-ray film without the need for fluorography.

Electrophoretic Mobility Shift Assays

Mobility shift assays were carried out with approximately 0.5 ng of in vitro-transcribed uniformly 32P-labeled α-globin 3'UTR (~10,000 cpm) per reaction. Binding reactions were carried out with S100 (or S130) extract pretreated for 15 min on ice with 1 unit ACE-RNase inhibitor (5'–3') per 30 μl S100 extract and β-mercaptoethanol to 1%. Reactions were carried out in RNA binding buffer (RBB: 10 mM Tris–HCl, pH 7.5, 1.5 mM MgCl₂, 150 mM KCl, 2 μg/ml leupeptin) for 20 min at room temperature with 40 μg of S100 extract in a total volume of 15 μl. Unbound RNA was degraded with 1 U RNase T1 (Boehringer) and 10 ng RNase A (Sigma) for 10 min at room temperature. Nonspecific RNA–protein interactions were minimized by a 10-min competition with heparin at 5 mg/ml. The RNase-resistant complexes were resolved on a 6% polyacrylamide gel (60:1 acrylamide:bisacrylamide) in 0.5× TBE buffer at 8 V/cm (5, 8). A 20-nt region within the α-globin 3'UTR (nucleotides 41 to 60) appears to constitute a minimal α-complex binding domain since an RNA oligonucleotide of this sequence was shown to bind the α-complex proteins (24).

In an attempt to determine the minimal number of contiguous C residues that are required to bind αCP1 and αCP2 within the α-complex, competition experiments were performed using deoxyligonucleotides with defined stretches of cytidylate residues. As shown in Fig. 2, an oligonucleotide with 16 C
residues can efficiently compete for the α-complex, suggesting that an oligonucleotide of this size can efficiently bind and sequester αCP1 and αCP2 (compare lanes 3 and 4). Competition experiments using 16-mer oligonucleotides containing a defined number of contiguous C residues ranging from 5 (lane 5) to 9 (lane 9) flanked by random sequences indicate that 9 contiguous C residues are sufficient to efficiently compete for the α-complex. Therefore, αCP1 and αCP2 require 9 C residues in a row for efficient binding. Whether C residues are mandatory at all nine positions or other nucleotides can be tolerated is unknown. However, direct binding of αCP1 and αCP2 to C-rich regions within the poliovirus 5' UTR and the 15-lipoxygenase 3' indicates that substitutions are possible (17–19).

Northwestern Analysis

An interesting property of αCP1 and αCP2 is their ability to renature into a structure competent of binding oligo(C) following SDS–PAGE (5). This property has been used as an assay to purify these proteins (8) as well as demonstrate that the predominant poly(C) binding activity is contained within the first and third KH domains of both αCP1 and αCP2 (22). The following method of binding 32P-labeled nucleic acid to protein immobilized on nitrocellulose membrane was adapted from Matunis et al. (7) with minor modifications. Proteins were separated on 12.5% SDS–PAGE and electroblotted onto nitrocellulose membrane for 1 h at 150 mA in transferr buffer (50 mM Tris, 14.5 mM glycine, 20% methanol). The transferred proteins were renatured by gentle rocking in Northwestern (NW) buffer (10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1× Denhardt’s solution, 1 mM DTT) for 1 h at room temperature. The blot was probed using 10⁸ cpm of 5'-end-labeled poly(C) (Sigma) per lane in NW buffer containing 10 ng/ml Escherichia coli tRNA for 1 h. It was then washed for 15 min in NW buffer containing 1 mg/ml heparin, followed by two additional 15-min washes in the same buffer. Heparin competition is critical since it minimizes the npspecific interactions that can be detected. The blot was then air-dried and exposed to X-ray film (5, 8).

Isolating RNAs Bound by αCP1 and αCP2

Multiple approaches can be used to determine if a given RNA is bound by a protein of interest. One approach is to immobilize the protein by either an antibody or as a fusion protein (e.g., GST fusion protein on glutathione beads) and isolate RNA bound to it. Bound RNA is isolated and detected by autoradiography if labeled exogenous RNA is used or by reverse transcription polymerase chain reaction (RT-PCR) if endogenous RNA is used instead. The first method describes immunocoprecipitation of an epitope-tagged αCP1 or αCP2 expressed in cells with a uniformly labeled α-globin 3'UTR. The second method describes a GST fusion protein copurification of RNA from cell extract.

Immuno-copurification of RNA

Immunoprecipitation of αCP1 and αCP2 was carried out under conditions that maintain RNA-protein interactions to enable copurification of bound RNA. Myc epitope-tagged αCP1 or αCP2 was expressed in 293T cells, a modified human embryonic kidney 293 cell line expressing the SV40 large T antigen (25). The presence of the T antigen enables replication of plasmids containing the SV40 origin of replication. Furthermore, on cell division, the plasmid is likely to be passed on to each daughter cell, resulting in further propagation. Therefore the advantages of using 293T cells include its high transfection efficiency (>50%), high copy plasmid per cell,
and high protein levels produced from genes encoded on the transfected plasmid.

Transfections were carried out in 10-cm² plates of 293T cells using standard calcium phosphate coprecipitation. The next day, transfected cells were split 1:4, and 2 days later, S100 extract was isolated as described above using scaled down conditions. One hundred micrograms of S100 extract pretreated with ACE-RNase inhibitor and β-mercaptoethanol was incubated with 100,000 cpm of in vitro-generated 32P-labeled 3′UTR RNA or a control mutant 32P-labeled 3′UTR RNA that is unable to interact with the α-complex proteins [αH19 in (5)] and thus should not immuno-copurify. Binding reactions were carried out in RBB for 20 min at room temperature followed by a 10-min incubation with heparin at 2.5 mg/ml as a nonspecific competitor. The myc-tagged αCP1 or αCP2 proteins and accompanying complex were immunopurified with the 9E10 anticyclosin antibody (26). Immunoprecipitations were carried out in RBB with 4 μl of 9E10 antibody ascites fluid (−20 μg) bound to Staphylococcus aureus protein A-Sepharose beads (30 μl; Pharmacia) on a rocking platform at 4°C for 30 min in 250-μl reactions. Immune complexes were then pelleted with a brief spin, washed by rocking at 4°C for 10 min with RBB containing 0.1% Triton X-100 (v/v) and 1 mg/ml heparin, and then rinsed four times with the same buffer and once with RBB only. The RNA–protein complex was disrupted by boiling for 3 min in 50 μl TES (10 mM Tris, pH 7.5, 1 mM EDTA, 1% SDS), followed by PCR with primers to amplify either the 3′UTR of α-globin or GAPDH. The α-globin 3′UTR was amplified using Taq polymerase (Promega) with the following buffer: 20 mM (NH₄)₂SO₄, 70 mM Tris–HCl, pH 8.8, 2 mM MgCl₂, 1 mM DTT, 1 mg/ml BSA, 1% Triton X-100, 10% dimethyl sulfoxide, and 2 mM of eachdNTP using the following primers: 5′-GCTGGAGCCCTCGGTACG-3′ and 5′-TTTTTGCCGGCCACTCAGACTTT-3′. PCR of GAPDH was carried out with Vent polymerase (NEB) with the manufactures’ buffer using the following primers: 5′-TGGACTGTGGTCATGAGTCC-3′ and 5′-ACCATGGAAAGGCTTGAGG-3′. PCR amplifications were carried out with 200 ng of each primer for 30 cycles at 92°C for 30 min, 52°C for 45 min, and 72°C for 40 min. As shown in Fig. 3, the α-globin mRNA specifically copurified with the GST–αCP1 protein but not the GST domain alone (compare lanes 1 and 2). Furthermore, a control mRNA for GAPDH did not copurify with either protein (lanes 4 and 5) even though GAPDH mRNA can be amplified from total K562 S130 extract under these conditions (lane 6). Although S130 extract was used for our purposes, total cell extract could have also been

Fusion Protein Copurification of RNA

A GST fusion protein could be used to copurify cellular mRNA bound to it. The GST fusion protein is first bound to glutathione beads and then allowed to form an RNP complex on incubation with cell extract containing protein and RNA. Isolation of the fusion protein enables cosolubilization of endogenous RNA bound to the protein. The following method details the use of a GST–αCP1 fusion protein to specifically purify the α-globin mRNA from K562 cell extract.

The GST–αCP1 fusion protein was expressed in E. coli BL21. Cells were disrupted by sonication according to the manufacturer (Pharmacia) using PBS with 0.5% Triton X-100. The extract was treated with micrococal nuclease as described above to eliminate bacterial RNA. Approximately 100 μg of GST fusion protein was bound to 20 μl GST beads in a total volume of 1 ml in RBB with 0.5% Triton X-100 (RBB/0.5% TX) and 2 μg/ml leupeptin and 0.5% (v/v) aprotinin at 4°C for 15 min. Unbound protein was removed with four 1-ml washes in RBB/0.5% TX and once in RBB. The washed beads (which carry the fusion protein of interest) were resuspended in 350 μl of RBB. Two hundred fifty micrograms of cytoplasmic S130 extract which contains cytoplasmic proteins and mRNAs was pre-cleared with 20 μl of glutathione Sepharose beads to remove background RNA that “binds” to either the GST domain or glutathione Sepharose beads. Incubation of the precleared S130 extract to the washed beads above was carried out at 4°C for 1 h followed by a wash in RBB/0.1% TX. RNA interacting nonspecifically with the fusion protein was competed off with a wash in RBB containing 1 mg/ml heparin for 10 min at 4°C. The beads were subsequently rinsed four times in RBB/0.1% TX to remove unbound RNA and the RNA was isolated from the drained beads by boiling for 3 min in 200 μl TE/1% SDS. The RNA was then phenol/ chloroform (1:1)-extracted, chloroform-extracted twice, ethanol-precipitated with 20 μg glycogen (Boehringer-Mannheim), and washed with 70% ethanol. The dried RNA was resuspended in 10 μl diethyl pyrocarbonate-treated H₂O and heated to 65°C for 5 min.

The bound RNAs were detected by a reverse transcriptase reaction using oligo(dT) (Stratagene) followed by PCR with primers to amplify either the 3′UTR of α-globin or GAPDH. The α-globin 3′UTR was amplified using Taq polymerase (Promega) with the following buffer: 20 mM (NH₄)₂SO₄, 70 mM Tris–HCl, pH 8.8, 2 mM MgCl₂, 1 mM DTT, 1 mg/ml BSA, 1% Triton X-100, 10% dimethyl sulfoxide, and 2 mM of eachdNTP using the following primers: 5′-GCTGGAGCCCTCGGTACG-3′ and 5′-TTTTTGCCGGCCACTCAGACTTT-3′. PCR of GAPDH was carried out with Vent polymerase (NEB) with the manufactures’ buffer using the following primers: 5′-TGGACTGTGGTCATGAGTCC-3′ and 5′-ACCATGGAAAGGCTTGAGG-3′.
used. Another modification to this procedure could be the expression of the GST fusion protein in an appropriate eukaryotic cell, followed by copurification of bound mRNA to the fusion protein in vivo. This approach would bypass the need for bacterially expressed GST fusion protein.

Isolation of α-Complex Proteins

The αCP1 and αCP2 proteins are a subset of the α-complex proteins that form on the α-globin 3′UTR and are involved in the stability of this mRNA (5, 8). Under the assay conditions described below, at least six distinct protein or protein families appear to be present in the α-complex. αCP1 and αCP2 constitute one such family of proteins. The second is the AUF1/hnRNP D family of proteins (27), which was originally identified as a component involved in the rapid turnover of c-myc mRNA (28). The AUF1 proteins are described in more detail by Wilson and Brewer (29) in this issue and are not discussed further here. At least four additional proteins of apparent molecular weights of 58, 55, 50, and 28 are also present in the α-complex. Whether all these proteins are present simultaneously within the complex or some of them are mutually exclusive is still unclear. Determination of the various proteins within the complex was made by isolating the α-complex proteins from a native electrophoretic mobility shift assay, and then directly resolving and visualizing them by SDS–PAGE.

A modified version of the electrophoretic mobility shift assay described above was set up with 500 ng of unlabeled α3′UTR RNA and 1.5 mg K562 S130 extract in a total volume of 160 μl. The α-complex was allowed to form for 40 min in RBB and treated with RNase solution (40 ng RNase A and 4 units RNase T1) for 10 min followed by competition with 200 μg heparin. A 5-μl aliquot of the α-complex formed on 32P-labeled α3′UTR was also included to allow visualization of the complex, which was resolved on a 6% native polyacrylamide gel (as described above). The wet gel was exposed to X-ray film overnight at 4°C and the complex was excised using the autoradiograph as a guide. A control lane was included that contained only S130 protein extract and no addition of α-globin mRNA. To minimize the possibility that a residual amount of the α-complex might form in the control lane due to the presence of endogenous α-globin mRNA in the K562 S130 extract, the extract was treated with micrococcal nuclease (400 U/ml) and RNase A (35 μg/ml) at 30°C for 30 min prior to resolution on the native polyacrylamide gel. The excised gel slice containing the α-complex was soaked in 2× SDS–PAGE sample buffer for 5 min to disrupt the RNP complex and allow association of the proteins with SDS. The gel slice was directly overlaid onto a 12.5% SDS–PAGE well and proteins were electrophoresed into the stacking gel at 100 V with constant current for 1 h and resolved in the running gel for 4 h at 150 V. Proteins were transferred onto nitrocellulose with a Hoefer Semi-Phor blotter at 150 mA for 1 h and visualized by Ponceau S staining (0.5% w/v) for 10 min. A comparison of protein bands present in the α-complex lane with that of the comigrating protein-only lane allowed for the identification of the α-complex specific proteins (27). The blot could be destained with several rinses of H2O prior to use in Western analysis (27).

**CONCLUDING REMARKS**

The methods presented in this report have been described specifically for the αCP1 and αCP2 proteins. However, these applications are broad and can be applied to any RNA-binding protein. Ribohomopolymer binding is a good first approximation in determining whether an unknown protein is competent of binding RNA. However, care should be taken in interpreting ribohomopolymer binding studies since the homopolymers represent an artificial substrate [except for poly(A)]. In particular, binding conditions should be stringent enough to minimize simple electrostatic interactions which...
could occur under low-salt conditions. We routinely use buffers containing > 150 mM KCl (or NaCl) along with a nonionic detergent to minimize nonspecific interactions. This is most critical with binding assays to poly(G) which appear to be promiscuous in their protein interaction at low stringencies.

Isolation of RNA bound to an RNA-binding protein could provide valuable insights into the possible function of a protein. If a target mRNA is suspected, isolation of the protein by immunoprecipitation or purification could determine whether the RNA copurifies. A defined 32P-labeled RNA could be used and detected directly, or endogenous RNA can be isolated and detected by either RT-PCR or RNase Protection Assay. Unknown mRNA specifically bound by an RNA-binding protein can also be isolated and identified provided appropriate controls are used to eliminate false positives. This approach is described elsewhere (30).

Many mRNA processing events (including α-globin mRNA stability) are controlled by and/or occur within RNP complexes. Therefore, it is critical to identify the various protein components within a given complex. One straightforward way to accomplish this task is to directly isolate protein components by excising a gel slice containing the proteins from a native electrophoretic mobility shift assay and resolving them by SDS–PAGE. Elimination of the protein extraction step from the native gel prior to resolution by SDS–PAGE minimizes loss of protein. It is, however, critical to include a negative control that has protein only without any RNA to be able to distinguish protein bands that fortuitously comigrate with the RNP complex of interest. This approach provides an initial step in the identification and isolation of the protein components within an RNP complex under study.

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REFERENCES