

# Purification and RNA Binding Properties of the Polycytidylate-Binding Proteins $\alpha$ CP1 and $\alpha$ CP2

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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  $\alpha$ -globin mRNA is determined by elements in the 3' untranslated region that are bound by an RNP complex ( $\alpha$ -complex) which appears to control the erythroid-specific accumulation of  $\alpha$ -globin mRNA. The  $\alpha$ -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,  $\alpha$ -complex proteins 1 and 2 ( $\alpha$ CP1 and  $\alpha$ CP2). This article focuses on various methodologies for the detection and manipulation of  $\alpha$ CP1 and  $\alpha$ CP2 binding to RNA and details means of isolating and characterizing mRNA bound by these proteins to study mRNA turnover and its regulation. © 1999 Academic Press

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  $\alpha$ -globin mRNA is conferred by a pyrimidine-rich region in the 3' untranslated region (3'UTR) that forms a specific RNP complex ( $\alpha$ -complex) (4–6). The formation of the  $\alpha$ -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  $\alpha$ -complex,  $\alpha$ -complex proteins 1 and 2 ( $\alpha$ CP1 and  $\alpha$ CP2) (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).

$\alpha$ CP1 and  $\alpha$ CP2 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

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domains each.  $\alpha$ CP2 was originally reported by Hahm *et al.* (13) as a protein that copurified with a lymphocyte transcription factor. The presence of KH domains in  $\alpha$ CP2 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  $\alpha$ CP1 was initially reported by Aasheim *et al.* (15) as clone sub2.3, which had similarity to  $\alpha$ CP2 and also contained poly(C) binding activity. Leffers *et al.* (9) isolated human  $\alpha$ CP2 [referred to as poly(C)-binding protein 2, PCBP2, based on its binding activity]. A functional role for  $\alpha$ CP1 and  $\alpha$ CP2 was first reported by Kiledjian *et al.* (8). The poly(C) binding activity within the  $\alpha$ -globin mRNA-stabilizing  $\alpha$ -complex was purified and shown to be the  $\alpha$ CP1 and  $\alpha$ CP2 proteins. This finding therefore implicated these proteins in mRNA stability.  $\alpha$ CP1 and  $\alpha$ CP2 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  $\alpha$ CP1 and  $\alpha$ CP2 have been implicated in poliovirus replication and translation (16–18), while  $\alpha$ CP1 has also been implicated in the translational regulation of the 15-lipoxygenase mRNA (19).

The  $\alpha$ CP1 and  $\alpha$ CP2 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  $\beta\alpha\alpha\beta\alpha$  structure with three antiparallel  $\beta$  sheets on one surface positioned against three  $\alpha$  helices on another (20). An invariable Gly–X–X–Gly sequence, where the X is usually a positively charged amino acid, is contained within the loop between helices 1 and 2 and is thought to be essential for RNA binding (21). The first and third KH domains of both  $\alpha$ CP1 and  $\alpha$ CP2 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  $\alpha$ CP binding to RNA. It describes the purification of  $\alpha$ CP1 and  $\alpha$ CP2 from erythroid cells as well as the purification of recombinant bacterially expressed protein. It details methods to detect the RNA binding properties of  $\alpha$ CP1 and  $\alpha$ CP2, in addition to procedures to isolate RNA bound by these

proteins, and describes the isolation and resolution of an RNP complex containing these proteins.

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## DESCRIPTION OF METHODS

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### Purification of the $\alpha$ CP1 and $\alpha$ CP2 Proteins

Methods are presented below that describe the purification of endogenous  $\alpha$ CP1 and  $\alpha$ CP2 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  $\alpha$ CP1 and  $\alpha$ CP2 copurify and do not resolve when isolated from eukaryotic cells using the procedure described below. Recombinant protein must be used whenever purified  $\alpha$ CP1 or  $\alpha$ CP2 only are required. Although  $\alpha$ CP1 and  $\alpha$ CP2 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  $\mu$ g/ml leupeptin, and 0.5% aprotinin. 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  $-70^{\circ}\text{C}$ .

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 12,000*g* centrifugation and the supernatant was layered over the above lysis buffer containing 30% (w/v) sucrose. The sample was then centrifuged at 130,000*g* 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  $-70^{\circ}\text{C}$ .

#### *Purification of Cellular $\alpha\text{CP1}$ and $\alpha\text{CP2}$ 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  $\text{CaCl}_2$  at  $30^{\circ}\text{C}$  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 10,000*g* 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, 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-Sepharose (Pharmacia) column also equilibrated in HEG-40 at the same flow rate. The KCl concentration of the DEAE-Sepharose 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  $\mu\text{l}$  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  $\alpha\text{CP1}$  and  $\alpha\text{CP2}$  proteins eluted at approximately 800 mM KCl.

#### *Purification of Recombinant $\alpha\text{CP1}$ and $\alpha\text{CP2}$ Proteins*

Whenever recombinant proteins are required, the  $\alpha\text{CP1}$  and  $\alpha\text{CP2}$  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  $\alpha\text{CP1}$  and  $\alpha\text{CP2}$  using thrombin protease digestion because the protein was degrading. However, efficient isolation of bacterially expressed  $\alpha\text{CP1}$  and  $\alpha\text{CP2}$  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- $\alpha\text{CP1}$  fusion protein ( $\sim 70$  kDa) was overexpressed in *Escherichia coli* BL21 cells on induction with 0.2 mM isopropyl- $\beta$ -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  $4^{\circ}\text{C}$  on a nutator. Following cleavage, the supernatant containing cleaved  $\alpha\text{CP1}$  was collected (lane 4). The protease that contains a GST domain and the GST domain of the original GST- $\alpha\text{CP1}$  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-

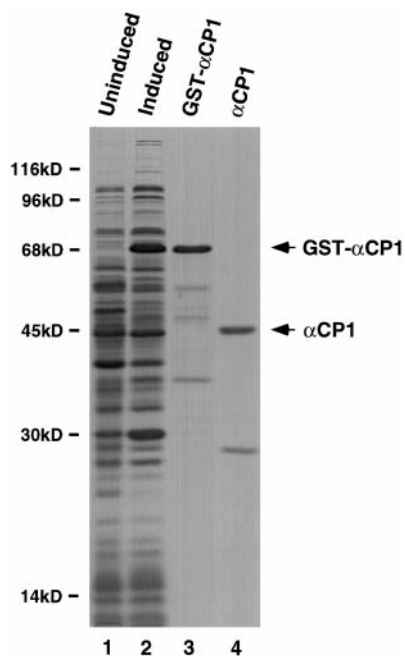
cated  $\alpha$ CP1 protein (lane 4). Separation of the full-length  $\alpha$ CP1 from the proteolytic products could be achieved by purification on a ssDNA column as described above.

### Testing $\alpha$ CP1 and $\alpha$ CP2 Binding to RNA

#### *Ribohomopolymer Binding Assays*

The first biochemical activity attributed to  $\alpha$ CP1 and  $\alpha$ CP2 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.  $\alpha$ CP1 and  $\alpha$ CP2 were transcribed and translated *in vitro* in the presence of [ $^{35}$ S]methionine (Amersham) using the TNT rabbit reticulocyte system (Promega Biotech) as per the manufacturer. Approximately  $10^5$  trichloroacetic acid-precipitable cpm of *in vitro*-produced protein was bound to 30  $\mu$ g of the ribonucleotide homopolymer-agarose beads (Sigma) in HB buffer (10 mM Tris-HCl, 250 mM NaCl,

1.5 mM MgCl<sub>2</sub>, 0.5% v/v Triton X-100, 2  $\mu$ g/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  $\mu$ l 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  $^{35}$ S and allows direct exposure of the dried gel onto X-ray film without the need for fluorography.



**FIG. 1.** Purification of  $\alpha$ CP1 using the pGex6p system. Total soluble extract from *E. coli* BL21 cells transformed with pGex6p- $\alpha$ CP1 prior to induction (lane 1) or after induction (lane 2) of fusion protein expression is shown. Lane 3 contains GST- $\alpha$ CP1 fusion protein purified on a glutathione-Sepharose column, and lane 4, the  $\alpha$ CP1 protein released after proteolytic cleavage. The smaller band present in lane 4 is most likely an  $\alpha$ CP1 proteolytic product. Molecular weight sizes are indicated on the left, and the positions of the GST- $\alpha$ CP1 fusion protein and  $\alpha$ CP1 protein are indicated by the arrows.

#### *Electrophoretic Mobility Shift Assays*

Mobility shift assays were carried out with approximately 0.5 ng of *in vitro*-transcribed uniformly  $^{32}$ P-labeled  $\alpha$ -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  $\mu$ l S100 extract and  $\beta$ -mercaptoethanol to 1%. Reactions were carried out in RNA binding buffer (RBB: 10 mM Tris-HCl, pH 7.5, 1.5 mM MgCl<sub>2</sub>, 150 mM KCl, 2  $\mu$ g/ml leupeptin) for 20 min at room temperature with 40  $\mu$ g of S100 extract in a total volume of 15  $\mu$ l. 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 $\times$  TBE buffer at 8 V/cm (5, 8). A 20-nt region within the  $\alpha$ -globin 3'UTR (nucleotides 41 to 60) appears to constitute a minimal  $\alpha$ -complex binding domain since an RNA oligonucleotide of this sequence was shown to bind the  $\alpha$ -complex proteins (24).

In an attempt to determine the minimal number of contiguous C residues that are required to bind  $\alpha$ CP1 and  $\alpha$ CP2 within the  $\alpha$ -complex, competition experiments were performed using deoxyoligonucleotides with defined stretches of cytidylate residues. As shown in Fig. 2, an oligonucleotide with 16 C

residues can efficiently compete for the  $\alpha$ -complex, suggesting that an oligonucleotide of this size can efficiently bind and sequester  $\alpha$ CP1 and  $\alpha$ CP2 (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  $\alpha$ -complex. Therefore,  $\alpha$ CP1 and  $\alpha$ CP2 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  $\alpha$ CP1 and  $\alpha$ CP2 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  $\alpha$ CP1 and  $\alpha$ CP2 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  $\alpha$ CP1 and

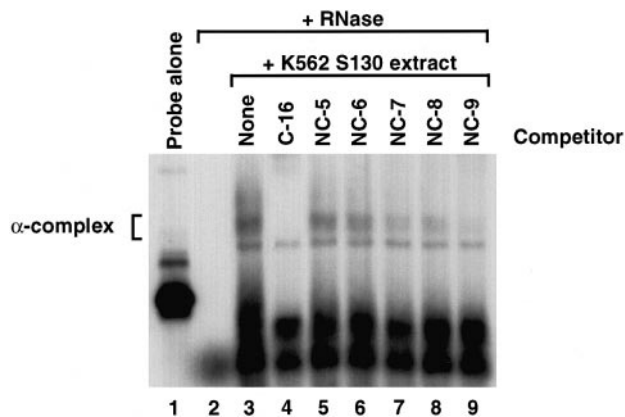
$\alpha$ CP2 (22). The following method of binding  $^{32}$ P-labeled nucleic acid to protein immobilized on nitrocellulose membrane was adapted from Matunis *et al.* (7) with minor modifications. Proteins were separated on 12.5% SDS-PAGE and electroblotted onto nitrocellulose membrane for 1 h at 150 mA in transfer 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 $\times$  Denhardt's solution, 1 mM DTT) for 1 h at room temperature. The blot was probed using  $10^5$  cpm of 5'-end-labeled poly(C) (Sigma) per lane in NW buffer containing 10 mg/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 nonspecific interactions that can be detected. The blot was then air-dried and exposed to X-ray film (5, 8).

### Isolating RNAs Bound by $\alpha$ CP1 and $\alpha$ CP2

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 immunoprecipitation of an epitope-tagged  $\alpha$ CP1 or  $\alpha$ CP2 expressed in cells with a uniformly labeled  $\alpha$ -globin 3'UTR. The second method describes a GST fusion protein copurification of RNA from cell extract.

### Immuno-copurification of RNA

Immunoprecipitation of  $\alpha$ CP1 and  $\alpha$ CP2 was carried out under conditions that maintain RNA-protein interactions to enable copurification of bound RNA. Myc epitope-tagged  $\alpha$ CP1 or  $\alpha$ CP2 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,



**FIG. 2.** Competition of the  $\alpha$ -complex with oligodeoxynucleotides. An electrophoretic mobility shift assay using uniformly  $^{32}$ P-labeled  $\alpha$ -globin 3'UTR mRNA and K562 cytoplasmic S130 extract is shown. The first two lanes contain the RNA probe in the absence and presence of RNase, respectively. The subsequent lanes show the RNase-resistant  $\alpha$ -complex formed on the 3'UTR in the presence of 250 ng of various 16-base competitor oligodeoxynucleotides. Lane 4, the 16-mer, contains only cytidylate residues, while in the subsequent lanes 16-mers with a defined stretch of cytidylate surrounded by random sequences are used. The stretch of C residues ranged from 5 (NC-5) to 9 (NC-9) residues. Migration of the  $\alpha$ -complex is as indicated.

and high protein levels produced from genes encoded on the transfected plasmid.

Transfections were carried out in 10-cm<sup>2</sup> 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  $\beta$ -mercaptoethanol was incubated with 100,000 cpm of *in vitro*-generated <sup>32</sup>P-labeled  $\alpha$ 3'UTR RNA or a control mutant <sup>32</sup>P-labeled  $\alpha$ 3'UTR RNA that is unable to interact with the  $\alpha$ -complex proteins [ $\alpha$ H19 in (5)] and thus should not immunocopurify. 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  $\alpha$ CP1 or  $\alpha$ CP2 proteins and accompanying complex were immunopurified with the 9E10 anti-myc antibody (26). Immunoprecipitations were carried out in RBB with 4  $\mu$ l of 9E10 antibody ascites fluid (~20  $\mu$ g) bound to *Staphylococcus aureus* protein A-Sepharose beads (30  $\mu$ l; Pharmacia) on a rocking platform at 4°C for 30 min in 250- $\mu$ l 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  $\mu$ l TES (10 mM Tris, pH 7.5, 1 mM EDTA, 1% SDS), and phenol/chloroform-extracted. The RNA was ethanol-precipitated, resuspended in 80% formamide, resolved on a 7 M urea/6% polyacrylamide gel, and visualized by autoradiography (8).

#### *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 coisolation of endogenous RNA bound to the protein. The following method details the use of a GST- $\alpha$ CP1 fusion protein to specifically purify the  $\alpha$ -globin mRNA from K562 cell extract.

The GST- $\alpha$ CP1 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 micrococcal nuclease as described above to eliminate bacterial RNA. Approximately 100  $\mu$ g of GST fusion pro-

tein was bound to 20  $\mu$ l GST beads in a total volume of 1 ml in RBB with 0.5% Triton X-100 (RBB/0.5% TX) and 2  $\mu$ g/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  $\mu$ l of RBB. Two hundred fifty micrograms of cytoplasmic S130 extract which contains cytoplasmic proteins and mRNA was pre-cleared with 20  $\mu$ l of glutathione Sepharose beads to remove background RNA that "binds" to either the GST domain or glutathione Sepharose beads. Incubation of the pre-cleared 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  $\mu$ l TE/1% SDS. The RNA was then phenol/chloroform (1/1)-extracted, chloroform-extracted twice, ethanol-precipitated with 20  $\mu$ g glycogen (Boehringer-Mannheim), and washed with 70% ethanol. The dried RNA was resuspended in 10  $\mu$ l diethyl pyrocarbonate-treated H<sub>2</sub>O 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  $\alpha$ -globin or GAPDH. The  $\alpha$ -globin 3'UTR was amplified using *Taq* polymerase (Promega) with the following buffer: 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 70 mM Tris-HCl, pH 8.8, 2mM MgCl<sub>2</sub>, 1 mM DTT, 1 mg/ml BSA, 1% Triton X-100, 10% dimethyl sulfoxide, and 2 mM of each dNTP using the following primers: 5'-GCTGGAGCCTCGGTAGCCGT-3' and 5'-TTTT-TGCCGCCCACTCAGACTTT-3'. PCR of GAPDH was carried out with Vent polymerase (NEB) with the manufacturer's buffer using the following primers: 5'-TGGACTGTGGTCATGAGTCC-3' and 5'-ACCATGGAGAAGGCTGGGGC-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  $\alpha$ -globin mRNA specifically copurified with the GST- $\alpha$ CP1 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

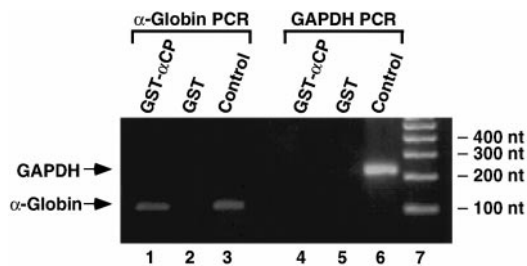
used. Another modification to this procedure could be the expression of the GST fusion protein in an appropriate eukaryotic cell, followed by copurification of bound mRNA to the fusion protein *in vivo*. This approach would bypass the need for bacterially expressed GST fusion protein.

### Isolation of $\alpha$ -Complex Proteins

The  $\alpha$ CP1 and  $\alpha$ CP2 proteins are a subset of the  $\alpha$ -complex proteins that form on the  $\alpha$ -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  $\alpha$ -complex.  $\alpha$ CP1 and  $\alpha$ CP2 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  $\alpha$ -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  $\alpha$ -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  $\alpha$ 3'UTR RNA and 1.5 mg K562 S130 ex-

tract in a total volume of 160  $\mu$ l. The  $\alpha$ -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  $\mu$ g heparin. A 5- $\mu$ l aliquot of the  $\alpha$ -complex formed on  $^{32}$ P-labeled  $\alpha$ 3'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  $\alpha$ -globin mRNA. To minimize the possibility that a residual amount of the  $\alpha$ -complex might form in the control lane due to the presence of endogenous  $\alpha$ -globin mRNA in the K562 S130 extract, the extract was treated with micrococcal nuclease (400 U/ml) and RNase A (35  $\mu$ g/ml) at 30°C for 30 min prior to resolution on the native polyacrylamide gel. The excised gel slice containing the  $\alpha$ -complex was soaked in 2 $\times$  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  $\alpha$ -complex lane with that of the comigrating protein-only lane allowed for the identification of the  $\alpha$ -complex specific proteins (27). The blot could be destained with several rinses of H<sub>2</sub>O prior to use in Western analysis (27).



**FIG. 3.**  $\alpha$ -globin mRNA specifically copurifies with the  $\alpha$ -complex. GST- $\alpha$ CP1 protein bound to glutathione-Sepharose beads was incubated with S130 K562 extract. Copurifying RNA was isolated following extensive washes and analyzed by RT-PCR using  $\alpha$ -globin (lanes 1–3)- or GAPDH (lanes 4–6)-specific primers. Lanes 1 and 4 are RT-PCR products from RNA copurified with GST- $\alpha$ CP1, while lanes 2 and 5 are products from RNA copurified with a control GST protein. Lanes 3 and 6 are RT-PCR products from total K562 RNA. The size markers are as indicated.

### CONCLUDING REMARKS

The methods presented in this report have been described specifically for the  $\alpha$ CP1 and  $\alpha$ CP2 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  $^{32}$ P-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  $\alpha$ -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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