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Finding the right RNA: Identification of cellular mRNA substrates for RNA-binding proteins

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ABSTRACT

Defects in RNA-binding proteins have been implicated in human genetic disorders. However, efforts in understanding the functions of these proteins have been hampered by the inability to obtain their mRNA substrates. To identify cognate cellular mRNAs associated with an RNA-binding protein, we devised a strategy termed isolation of specific nucleic acids associated with proteins (SNAAP). The SNAAP technique allows isolation and subsequent identification of these mRNAs. To assess the validity of this approach, we utilized cellular mRNA and protein from K562 cells and αCP1, a protein implicated in α-globin mRNA stability, as a model system. Immobilization of an RNA-binding protein with the glutathione-S-transferase (GST) domain enables isolation of mRNA within an mRNP context and the identity of the bound mRNAs is determined by the differential display assay. The specificity of protein–RNA interactions was considerably enhanced when the interactions were carried out in the presence of cellular extract rather than purified components. Two of the mRNAs specifically bound by αCP1 were mRNAs encoding the transmembrane receptor protein, TAPA-1, and the mitochondrial cytochrome c oxidase subunit II enzyme, coxII. A specific poly(C)-sensitive complex formed on the TAPA-1 and coxII 3′ UTRs consistent with the binding of αCP1. Furthermore, direct binding of purified αCP proteins to these 3′ UTRs was demonstrated and the binding sites determined. These results support the feasibility of the SNAAP technique and suggest a broad applicability for the approach in identifying mRNA targets for clinically relevant RNA-binding proteins that will provide insights into their possible functions.

Keywords: αCP1; coxII; poly(C)-binding; RNA substrate; SNAAP technique; TAPA-1

INTRODUCTION

A number of different human genetic disorders are associated with a defect in an RNA-binding protein (Levine et al., 1993; Ma et al., 1993; Buckanovich et al., 1996; Siomi et al., 1996; Timchenko et al., 1996; Mueller-Pillasch et al., 1997). Although, these proteins play a role in the processing and turnover of RNA, relatively little is known about their function. A major challenge is to identify the RNA substrates of these proteins. The current methodology involves the use of SELEX (systemic evolution of ligands by exponential enrichment; Tuerk & Gold, 1990). In this assay, a pool of RNA bound by an RNA-binding protein is subjected to multiple rounds of selection and PCR amplification to determine the strongest RNA substrate sequence. Although many variations of this approach have been developed and successfully utilized, a limitation is that short stretches of RNA are used as a substrate and the high affinity sequence obtained might not exist in vivo. In most cases, only RNA substrates that are bound directly by the RNA-binding protein are identified. An alternative approach would be one that utilizes intact endogenous cellular mRNA within its natural mRNP context.

In this report, we describe a strategy that is a combination of an mRNA copurification assay and the differential display technology. The combination of these existing methodologies was termed isolation of specific nucleic acids associated with proteins (SNAAP) and it allows the identification of natural mRNA substrates for any given RNA-binding protein. The technique uses an extract containing a pool of cellular RNAs as potential substrates of the protein of interest. This approach has the advantage of maintaining the protein–RNA interaction that occurs in the natural cell extract environment where both mRNAs as well as additional proteins that might be necessary to form an mRNP complex are present.

To test whether this approach is feasible, we used the α complex protein 1 (αCP1), an RNA-binding protein that interacts with and stabilizes the human α-globin mRNA (Kiledjian et al., 1995; Wang et al., 1999) as a

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model system. The stability of the 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; Weiss & Liebhaber, 1994, 1995; Wang et al., 1995) and includes the poly(C)-binding αCP1 and αCP2 proteins (Kiledjian et al., 1995). The αCPs are highly homologous ($>$90% similar) proteins encoded by two distinct genes (Tommerup & Leffers, 1996). Each protein contains three KH domains, a 60-amino-acid-long motif essential for RNA binding. The αCPs have a functional role in the stability of the human α-globin mRNA (Kiledjian et al., 1995; Wang et al., 1999) but, interestingly, they appear to be ubiquitously expressed (Aasheim et al., 1994; Leffers et al., 1995). A minimal α-complex binding site in the α-globin mRNA was identified, and database searches revealed three additional mRNAs, tyrosine hydroxylase, α(I)-collagen, and 15-lipoxygenase, that form ribonucleoprotein complexes that include αCP1 (Holcik & Liebhaber, 1997). Therefore, αCP1 may be a general determinant in the stabilization of eukaryotic mRNAs. To broaden the repertoire of mRNA substrates for αCP1 beyond the database search matches and to validate the new approach, we employed the SNAAP technique using the αCP1 protein.

RESULTS

Copurification of specific RNA interacting with αCP1

Our interest was to identify cellular target mRNA with which an RNA-binding protein can associate and potentially regulate. At least three different modes of action can be envisioned by which an RNA-binding protein can associate with an mRNA (Fig. 1). The RNA-binding protein can bind directly to an RNA as depicted in Figure 1A. Alternatively, an RNA-binding protein might require an interaction with a second protein that could or could not be an RNA-binding protein. The interaction of the two proteins now creates a suitable RNA-binding domain that can contact RNA (Fig. 1B). Third, the RNA-binding protein might not contact RNA directly; rather, it might associate with another RNA-binding protein that is bound to the RNA (Fig. 1C). The same RNA-binding protein might contact different RNAs by one or more of the ways described above. RNA substrates that are bound directly or indirectly by the RNA-binding protein are still true substrates and are potentially regulated by this protein regardless of the way in which they are being contacted.

As an initial step in isolating specific cellular RNA bound by an RNA-binding protein, we utilized the αCP1 protein and α-globin mRNA as a model system. αCP1 is one of the major constituents of the α-complex, which appears to be a multiprotein complex involved in the stabilization of the α-globin mRNA (Wang et al., 1995, 1999; Kiledjian et al., 1997). Despite being an avid poly(C)-binding protein, purified αCP1 from K562 cells (or the highly homologous αCP2) is necessary but not sufficient for the formation of the α-complex (Kiledjian et al., 1995; Wang et al., 1995). To immobilize the RNA-binding protein onto a column matrix and permit direct isolation of the protein complex and any associated mRNA, the glutathione-S-transferase (GST) domain was fused to the αCP1 protein. GST-αCP1 was expressed in BL21 cells, purified using glutathione Sepharose beads, and then allowed to interact with K562 cytosolic S130 extract, which contains both proteins (250 μg) and mRNA (5 μg). RNAs nonspecifically bound to the fusion protein were eliminated with extensive washes in binding buffer followed by a heparin wash (see Materials and Methods). The mRNP complexes containing the αCP1 fusion protein were isolated by pelleting the Sepharose beads, and the specifically bound mRNA was extracted.

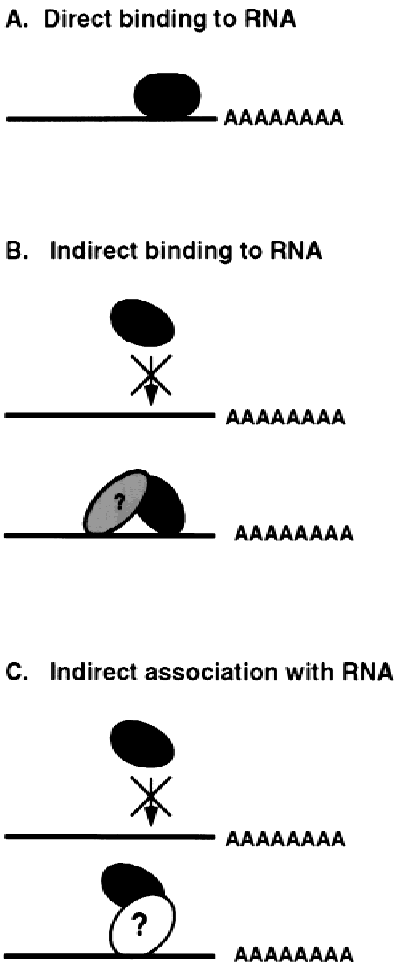

FIGURE 1. Interactions of an RNA-binding protein with RNA. **A**: Schematic diagram showing an RNA-binding protein that can bind RNA on its own. **B**: In this diagram, the RNA-binding protein cannot bind RNA on its own. Protein interactions are required that enable this RNA-binding protein to bind RNA. **C**: Alternatively, the RNA-binding protein might not contact the RNA directly but instead interact with a second, RNA-binding protein(s) that in turn binds to RNA.

To test the accuracy and specificity of the copurification step, we determined whether the α-globin mRNA copurified with GST-αCP1 when the fusion protein was incubated with K562 S130 extract. The presence of the α-globin mRNA in K562 cells was shown by reverse transcription and PCR (RT-PCR) amplification with α-globin specific primers (Fig. 2A, lane 1). A similar analysis of RNA copurified with the GST-αCP1 fusion protein revealed the presence of the α-globin mRNA (Fig. 2A, lane 3). Copurification of the α-globin mRNA was mediated through the αCP1 protein, as this mRNA did not copurify with the GST domain alone (Fig. 2A, lane 2). Because K562 cells express both α-globin and γ-globin, we tested whether the closely related and equally abundant γ-globin mRNA would copurify with the GST-αCP1 fusion protein. Reverse transcription of the copurified RNA and PCR amplification with γ-globin-specific primers demonstrates that the γ-globin mRNA did not copurify with the GST-αCP1 fusion protein (Fig. 2A, lane 6) nor with the GST domain (Fig. 2A, lane 5) despite its presence in K562 cells (Fig. 2A, lane 4). The lack of γ-globin mRNA was not a consequence of selective mRNA degradation during the reaction, as γ-globin mRNA was readily detected in the unbound supernatant (data not shown). The specific isolation of the α-globin but not the γ-globin mRNA with the GST-αCP1 fusion protein further underscores the specificity of the RNA copurification step of the assay. The inability of the αCP1 protein to bind and copurify the γ-globin mRNA is consistent with the inability of an αCP-dependent complex forming on the γ-globin 3′ UTR (data not shown).

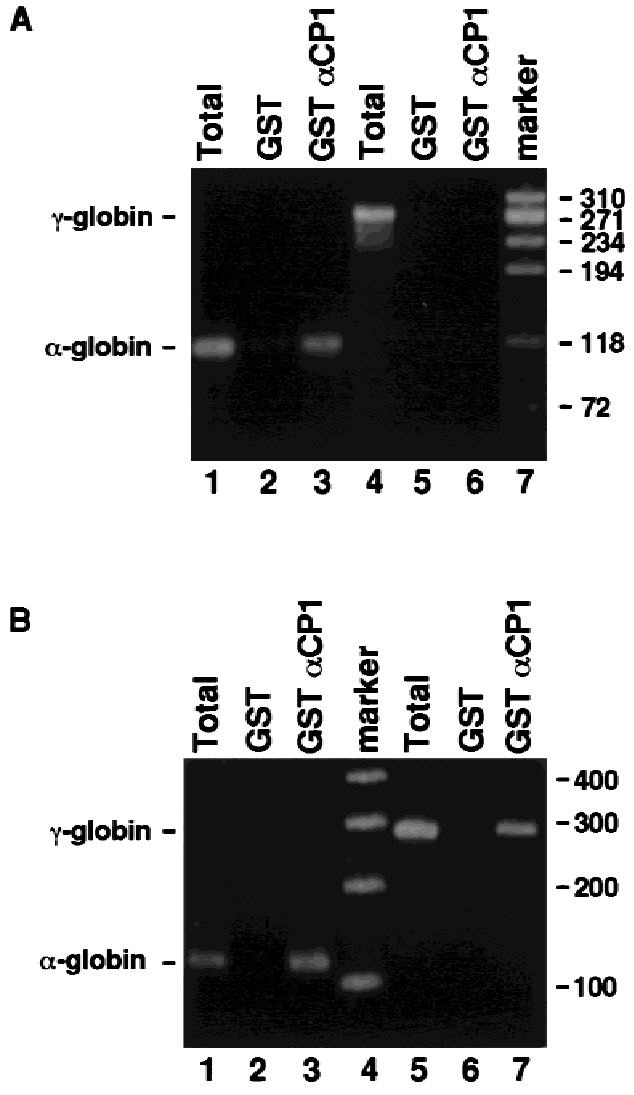

FIGURE 2. α-globin but not γ-globin mRNA specifically copurifies with the αCP1 protein in the presence of extract. **A**: RNAs copurifying with GST only or GST-αCP1 in the presence of K562 S130 extract containing 250 μg protein and 5 μg RNA were isolated, RT-PCR amplified with either α-globin (lanes 2 and 3) or γ-globin (lanes 5 and 6) specific primers. Total K562 RNA was reverse transcribed with oligo dT primers and used as a positive control for each set of primers (lanes 1 and 4). DNA molecular weight markers (lane 7) are shown on the right in nucleotides. **B**: RNA copurification reactions were carried out as described above except 5 μg RNA purified from S130 extract was used directly without cytosolic proteins. DNA molecular weight markers (lane 4) are shown on the right in nucleotides.

As a comparison, we tested the specificity of GST-αCP1 binding to purified mRNA in the absence of additional proteins. We did not expect to copurify the α-globin mRNA because αCP1 does not efficiently bind to it directly. In this experiment, instead of using the S130 extract that contains both cytosolic protein and RNA, the RNA component (5 μg) was isolated from an equivalent amount of S130 extract and used directly in the incubation reaction under the same buffer conditions. Surprisingly, the specificity of the copurified RNA was lost. RT-PCR amplification of this copurified RNA with specific primers revealed that α-globin mRNA copurified with the GST-αCP1 fusion protein (Fig. 2B, lane 3). However, the γ-globin mRNA also copurified with the GST-αCP1 (Fig. 2B, lane 7) suggesting that the specificity of the mRNA copurification was lost when only purified RNA was used. Despite the fact that the same amount of input RNA was used in all the copurification reactions of Figures 2A and 2B, 40% ($\sim$2 μg) of the input RNA was retained when the reactions were carried out with purified RNA. Conversely, only 0.02% ($\sim$1 ng) of the input RNA was retained by the αCP1 fusion protein when the interactions were carried out in the presence of cytosolic proteins. Therefore, these data indicate that in this copurification assay, the presence of the natural complement of proteins under physiologic salt conditions provides specificity for the RNA substrate interaction with the fusion RNA-binding protein.

Isolation of novel mRNA substrates bound by αCP1

Having demonstrated that the α-globin mRNA specifically copurifies with the fusion protein in the presence

of S130 extract, we initiated the isolation and identification of novel mRNAs bound to the αCP1 protein. mRNP complexes containing the GST-αCP1 fusion protein were isolated by pelleting the Sepharose beads, followed by extraction of the specifically bound mRNA. The identities of these mRNAs were subsequently determined by the differential display assay (Liang & Pardee, 1992). Briefly, RNAs copurifying with the GST-αCP1 fusion protein were reverse transcribed using one of three 3′ primers containing 11 T nucleotides followed by an A, C, or G nucleotide ($HT_{11}A$, $HT_{11}C$, or $HT_{11}G$, respectively). The first strand was then used in a PCR with one of the above three 3′ primers and eight of the possible 80 random 5′ primers (H-AP1 to H-AP80; GenHunter Corp.). Because only a subpopulation of mRNA binds to the αCP1 protein, multiple 5′ primers were used simultaneously. To control for nonspecific binding, RNAs copurifying with the GST domain only or a GST fusion protein with a different RNA-binding domain were RT-PCR amplified, and compared to GST-αCP1 copurifying RNA. The PCR products were resolved on a denaturing polyacrylamide gel.

A representative example of two differential display gels is illustrated in Figure 3 using a single 3′ primer with an anchored adenosine ($HT_{11}A$) and two different sets of 5′ primers. Primers H-AP1 through H-AP4 were used in Figure 3A simultaneously to amplify mRNA copurified with GST-αCP1. Primers H-AP5 through H-AP8 were used simultaneously in Figure 3B. The presence of mRNA in S130 extract was demonstrated by RT-PCR amplification of the isolated RNA (Figs. 3A and 3B, lanes 1). Messenger RNAs copurifying with GST,

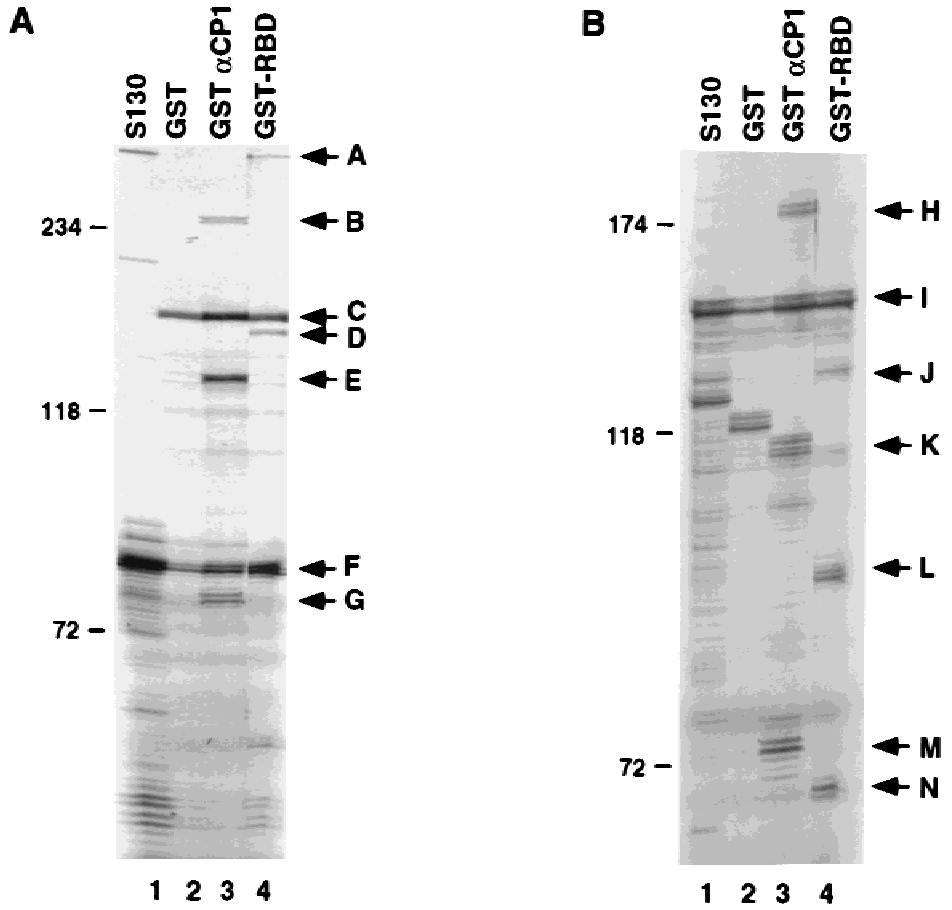

FIGURE 3. Isolation of specific cellular mRNAs bound by the αCP1 protein using SNAAP. Differential display PCR product profile of copurified mRNAs resolved by PAGE analysis. **A**: PCR reactions were carried out using the $HT_{11}A$ 3′ primer and the H-AP1 to H-AP4 5′ primers. Bands labeled B, E, and G represent PCR products from specific mRNAs that can interact with αCP1 (lane 3) but not with GST (lane 2) or a GST fusion to a different RNA-binding domain (GST-RBD; from the hnRNP U protein; lane 4). Bands A and D represent PCR products from specific mRNAs that can interact with GST-RBD. Bands C and F are PCR products from nonspecific mRNAs that appear to interact with all the proteins tested (lanes 2–4). The specific bands were excised from the gel, reamplified, cloned, and sequenced. Lane 1 contains S130 RNA that was RT-PCR amplified and used as a positive control. The numbers to the left are DNA size markers in nucleotides. **B**: PCR reactions were carried out as described above but using the $HT_{11}A$ 3′ primer and the H-AP5 to H-AP8 5′ primers. Bands H, K, and M are PCR products from mRNA that specifically associate with GST-αCP1 (lane 3). Bands J, L, and N are from mRNA that can specifically associate with GST-RBD (lane 4), and band I is a PCR product of a nonspecific mRNA. Identification of the specific PCR products, the fusion proteins used, and the size markers are as described in **A**.

GST-αCP1, or GST-RBD (the RNA binding domain of the hnRNP U protein; Kiledjian & Dreyfuss, 1992) were similarly RT-PCR amplified (Figs. 3A and 3B, lanes 2–4). To identify mRNAs that specifically interact with αCP1, the pattern obtained with GST-αCP1 was compared to that obtained with GST or GST-RBD. Bands labeled B, E, G, H, K, and M represent PCR products from specific mRNAs that can only interact with αCP1. Bands A, D, J, L, and N represent PCR products from mRNAs that interact only with the RBD of hnRNP U. In contrast, bands C, F, and I, which are present in all lanes, are PCR products from nonspecific mRNAs that can associate with all the proteins tested (Figs. 3A and 3B, lanes 2–4). Bands F and I, unlike band C, represent mRNAs that are abundant in the S130 extract. Several abundant RNAs are amplified in the S130 extract but fail to interact with αCP1 and, therefore, are not amplified in the GST-αCP1. On the contrary, the specific mRNAs amplified in the GST-αCP1 lanes (bands B, E, H, K, and M) are low-abundance mRNAs that are not readily amplified from S130 extract. A number of the PCR bands obtained appear as doublets on the acrylamide gel and most likely represent the complementary strands of the same PCR product, as they give identical sequence data.

Bands specifically bound by αCP1 (Figs. 3A and 3B, lanes 3) were excised from the acrylamide gel, reamplified with the same set of primers, cloned, and sequenced. The sequence from bands B, E, K, and M did not yield a match when compared to the GenBank (EMBL) database and these mRNAs remain unknown. The 81-nt sequence obtained from both G bands contained one of the 5′ random primer sequences at the 5′ end and the $HT_{11}A$ primer sequence at the 3′ end, as expected. The intervening sequence, when searched against the GenBank database, revealed an exact match to the terminal 60 nt of the TAPA-1 gene ending at the poly(A) tail. TAPA-1 is a 26-kDa integral membrane protein expressed on virtually all nucleated cells (Oren et al., 1990) but highly expressed on germinal-center B cells (Engel & Tedder, 1994). TAPA-1 is also known as the CD81 receptor. It is a member of the transmembrane pore integral membrane protein family, TM4SF, which is characterized by four hydrophobic membrane-spanning regions and three cysteine-containing short motifs (Wright & Tomlinson, 1994). A sequence search of band H revealed a 96% match to the terminal 159 nt of the cytochrome c oxidase subunit II (coxII) gene. This gene was also identified with the same set of four 5′ primers and the $HT_{11}G$ 3′ primer (data not shown). CoxII is one of three mitochondrial DNA-encoded subunits of respiratory Complex IV, which is involved in mitochondrial oxidative phosphorylation.

Several additional mRNAs were isolated that interacted specifically with αCP1 (data not shown). Four of these mRNAs were identified as the following: the mitochondrial NADH dehydrogenase 3 (ND3) gene, the ribosomal protein L27a gene, the palmitoylated erythrocyte membrane protein (MPP1) p55 gene, and the manganese superoxide dismutase (MnSOD) gene (Table 1). A total of seven mRNAs were isolated that did not have any homology to known genes in GenBank.

Although the α-globin mRNA can interact with the fusion protein and was specifically copurified (Fig. 2A), it was not among the mRNAs that associated with αCP1 above. The absence of this mRNA could be because only a subset of the copurifying RNAs was amplified, as only eight of the 80 5′ random primers that it would take theoretically to amplify the eukaryotic mRNA population (Liang et al., 1995) were used.

TAPA-1 and coxII mRNAs are specifically bound by αCP1

Copurification of the TAPA-1 and coxII mRNAs with αCP1 in the presence of extract was reconfirmed by RT-PCR using TAPA-1 and coxII specific primers (Fig. 4A). Primers specific for GAPDH were used as a control. The TAPA-1 mRNA is present in RNA copurified with the GST-αCP1 fusion protein (Fig. 4A, lane 7). Similarly, the coxII mRNA can be RT-PCR amplified from RNA copurified with GST-αCP1 (Fig. 4A, lane 3). Although the GAPDH mRNA is abundant, no signal for the GAPDH mRNA is present in the GST-αCP1 lane (Fig. 4A, lane 10) when GAPDH specific primers are used in the RT-PCR reaction. The absence of a GAPDH signal further underscores the specificity of the RNA–protein interaction in the presence of extract.

On the contrary, when the RNA–protein interaction was performed in the presence of naked RNA, specificity was once again lost. All three mRNAs, coxII, TAPA-1, and GAPDH, copurified with GST-αCP1 (Fig. 4B, lanes 3, 7, and 10, respectively). Therefore, the specificity of the RNA–protein interaction is greatly diminished when purified naked RNA is used in a column chromatography copurification assay under these conditions.

TABLE 1. Identification of mRNAs interacting with αCP1.

mRNAs bound by αCP1	Protein encoded	EMBL accession no.
ND3	mitochondrial NADH dehydrogenase subunit 3	X62996
coxII	mitochondrial cytochrome c oxidase subunit II	X55654
L27a	ribosomal protein of the large subunit	U14968
MPP1	palmitoylated erythrocyte membrane protein p55	M64925
MnSOD	manganese superoxide dismutase	S77127
TAPA-1	CD81 receptor	M33680

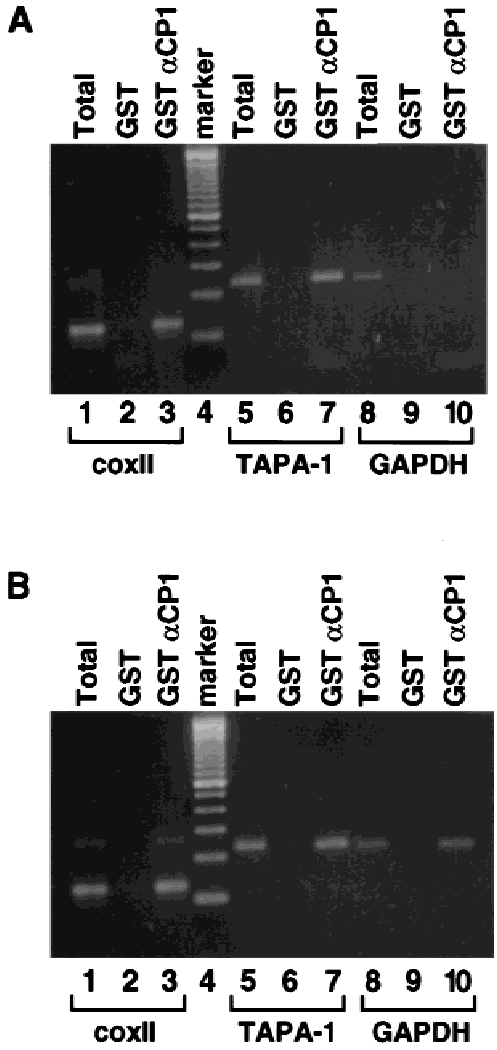

FIGURE 4. TAPA-1 and coxII but not GAPDH mRNAs copurify specifically with GST-αCP1 in the presence of extract. RNAs copurifying with GST only or GST-αCP1 in the presence of S130 extract (**A**) or pure RNA (**B**) were reverse transcribed as described in the legend to Figure 2. PCR amplifications were carried out with either coxII (lanes 2 and 3), TAPA-1 (lanes 6 and 7), or GAPDH specific primers (lanes 9 and 10). Total K562 RNA was reverse transcribed with oligo dT primers and used as a positive control for each set of primers (lanes 1, 5, and 8). The 100-bp ladder was used as a DNA molecular weight marker (lane 4).

Poly(C)-sensitive complexes form on the TAPA-1 and coxII mRNAs

The 535-nt-long 3′ UTR of the TAPA-1 mRNA is relatively C-rich, which is consistent with it being a substrate for αCP1 binding. We have demonstrated that a specific complex containing a poly(C)-binding activity can form on the terminal 350 nt of the TAPA-1 3′ UTR using a ^{32}P-labeled transcript in an electrophoretic mobility shift assay (EMSA) (Fig. 5A). Because αCP1 is an avid poly(C)-binding protein, poly(C) was used to compete the complex formed on the TAPA-1 3′ UTR mRNA. Figure 5A, lane 3, shows that this complex is sensitive to poly(C) competition because it was competed away by this ribohomopolymer, and a new, faster migrating complex was formed. This faster complex probably represents other proteins besides αCP1 that are still bound elsewhere on the large RNA probe. Poly(G) competition, as expected, did not disrupt the complex (Fig. 5A, lane 4). The observation that the complex formed is poly(C)-sensitive suggests the presence of αCP1 within this complex.

The coxII mRNA has a short 25 nt 3′ UTR and 15 of these nucleotides are C residues, making it a very strong potential binding site for αCP1. To determine whether a

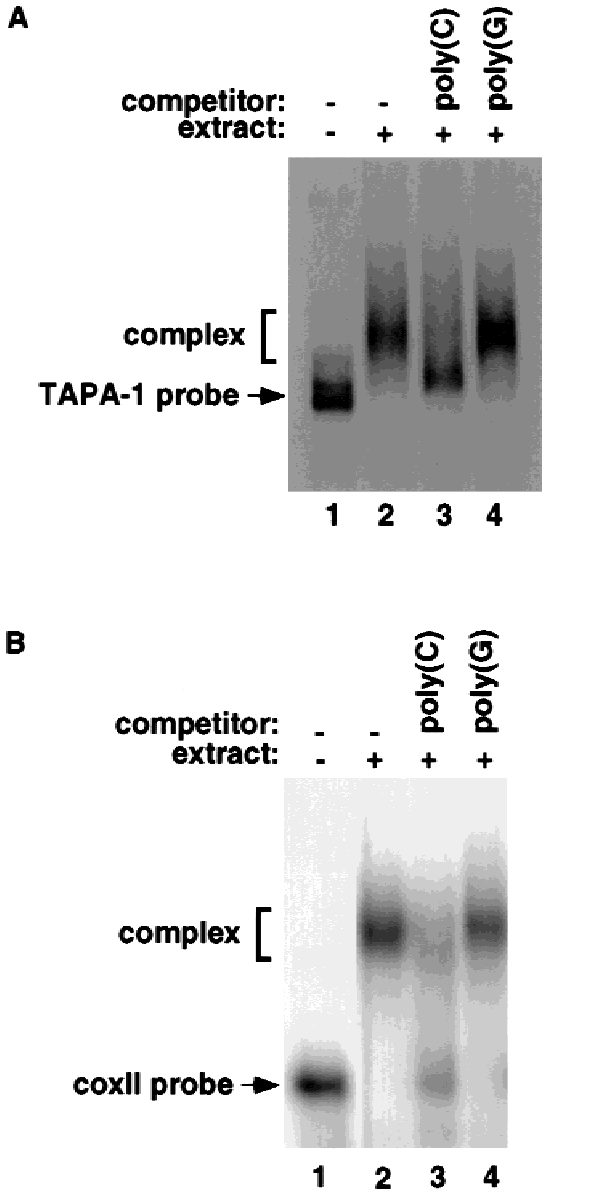

FIGURE 5. Presence of a poly(C)-binding activity in the TAPA-1 and coxII RNA complexes. An EMSA using ^{32}P-internally labeled TAPA-1 3′ UTR (**A**) or coxII RNA (**B**) and K562 cytoplasmic S130 extract. Lane 1 contains the RNA probe alone. The subsequent lanes show the complex formed on the 3′ UTR in the presence of 500 ng poly(C) (lane 3) or poly(G) (lane 4) competitor RNA. Migration of the complex is indicated.

complex containing αCP1 can also form on this mRNA, a ^{32}P-labeled transcript containing the 78 terminal nucleotides from the coxII coding region and its 3′ UTR was incubated with K562 S130 extract. A specific complex can form on this RNA (Fig. 5B, lane 2) in an EMSA. This complex is sensitive to poly(C) (Fig. 5B, lane 3) but not poly(G) competition (Fig. 5B, lane 4). Poly(C) addition fully competes this complex, suggesting that a poly(C)-binding protein is the primary protein that efficiently binds this RNA. Therefore, both the coxII and TAPA-1 mRNAs can form poly(C)-sensitive complexes with K562 S130 extract.

αCPs bind directly to the TAPA-1 and coxII mRNAs

To determine whether the poly(C)-binding activity contained within the TAPA-1 and coxII 3′ UTR complexes is that of the αCPs, αCPs purified from K562 cells were used in an EMSA to determine if they can bind directly to these RNAs. In Figure 6A, increasing amounts of the purified αCPs displayed binding to the TAPA-1 probe (lanes 2–4). The apparent upward shift in the complex when increasing amounts of protein are used might be because αCP1 and αCP2 can both form homo- and heterodimers (Gamarnik & Andino, 1997; our unpubl. observations). On the contrary, the same amounts of GST-RBD do not bind the TAPA-1 3′ UTR (Fig. 6A, lanes 5–7). These data are consistent with the absence of the TAPA-1 band (Fig. 3, band G) in the GST-RBD copurified RNA lane (Fig. 3A, lane 4).

To determine whether the αCPs can also bind the coxII probe, an EMSA was performed (Fig. 6B). The purified αCPs can also bind the coxII RNA probe (Fig. 6B, lanes 2–4). As expected, the GST-RBD cannot bind the coxII RNA in this EMSA assay (Fig. 6B, lanes 5–7) nor with the copurification assay in Figure 3B, lane 4.

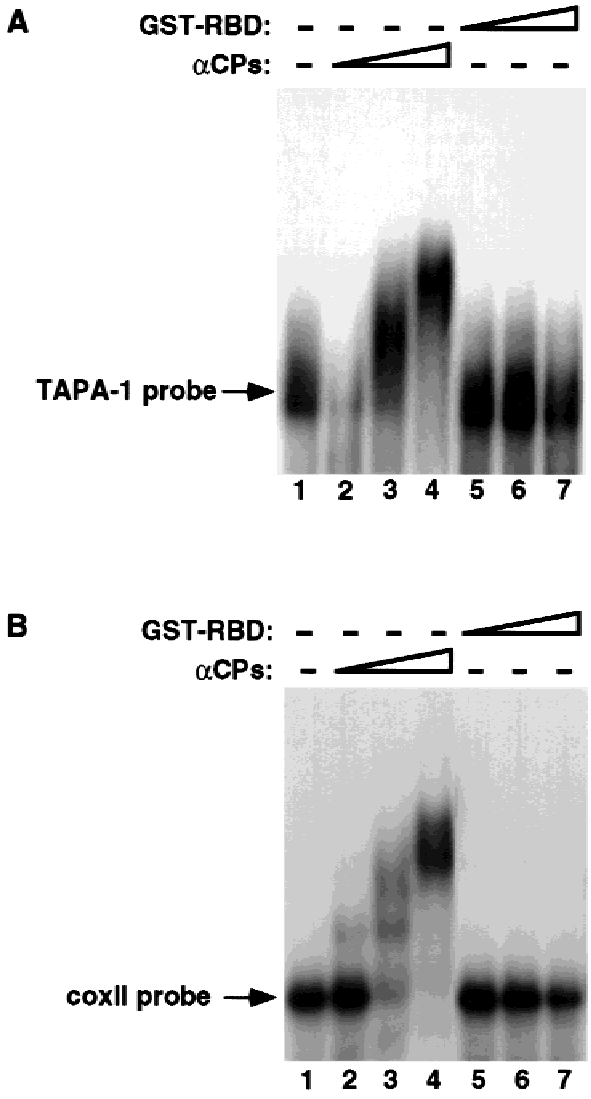

FIGURE 6. αCPs can bind directly to the TAPA-1 and coxII RNAs. An EMSA using ^{32}P-internally labeled TAPA-1 3′ UTR (**A**) or coxII RNA (**B**) and purified proteins. Lane 1 contains the RNA probe alone. Lanes 2–4 show the specific complexes formed with increasing amounts of purified αCP (25, 75, and 200 ng, respectively). No complex forms on these RNAs when incubated with increasing amounts (50, 100, or 300 ng) of GST-RBD (lanes 5–7, respectively).

Determining the αCP binding site in the TAPA-1 and coxII 3′ UTRs

We were able to demonstrate that two of the mRNAs that were identified as substrates for αCP1 using the SNAAP technique can indeed be bound by this protein. We then wanted to identify the αCP binding sites within these mRNAs. Examination of the sequence revealed two C-rich stretches, B1 and B2, within the 350 terminal nucleotides of the TAPA-1 3′ UTR that could potentially be αCP binding sites (Fig. 7A). We tested the binding of purified αCPs to these regions individually. Two deoxyoligonucleotides encompassing these sequences were used as probes in EMSAs (Fig. 7B). Purified αCPs can form a complex with the B2 probe (Fig. 7B, lane 2) that is competed by oligo dC and self oligonucleotide (Fig. 7B, lanes 3 and 4) as would be expected, but not by a nonspecific oligonucleotide (Fig. 7B, lane 5). Surprisingly, when the B1 probe was used in a similar experiment, no binding of the αCPs was observed (Fig. 7B, lanes 8–12) even though this region is also C-rich. Consistent with our previous observation, a complex was not formed with GST-RBD (Fig. 7B, lanes 6 and 12).

To determine whether the coxII 3′ UTR alone can bind the αCPs, a deoxyoligonucleotide consisting of the 25-nt sequence of the 3′ UTR was labeled and used in an EMSA (Fig. 8A). Purified αCPs were able to bind directly to this oligonucleotide (Fig. 8A, lane 2). The complex was sensitive to oligo dC and self competition (Fig. 8A, lanes 3 and 4) but insensitive to nonspecific oligonucleotide competition (Fig. 8A, lane 5).

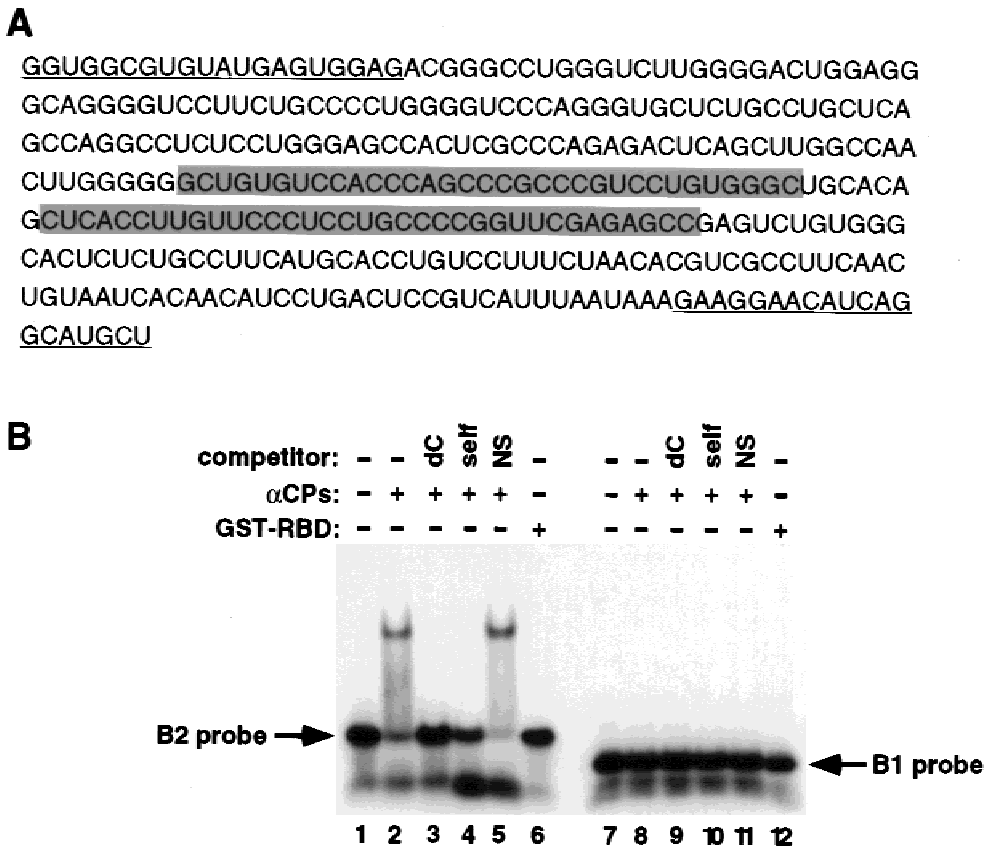

FIGURE 7. αCP binding site in the TAPA-1 3′ UTR. **A**: The sequence of the terminal 350 nt of the TAPA-1 3′ UTR. The underlined sequences correspond to oligonucleotides used to construct the riboprobe utilized in previous EMSAs. The shaded sequences correspond to the B1 and B2 regions, respectively, which were used as DNA oligonucleotide probes to determine the αCP binding site within the TAPA-1 3′ UTR. **B**: An EMSA using ^{32}P-5′ end labeled B2 (lanes 1–6) or B1 oligonucleotides (lanes 7–12) and purified αCP proteins. Lanes 1 and 7 contain the probe alone. Two hundred nanograms of purified αCPs were added to the probes (lanes 2–5 and 8–11) and the complex formed was competed with 15 pmol of the following oligonucleotides: oligo dC (lanes 3 and 9), a self oligonucleotide (lanes 4 and 10), and a nonspecific oligonucleotide (lanes 5 and 11). Two hundred and fifty nanograms of GST-RBD were added to the probe as a negative control (lanes 6 and 12).

As expected, GST-RBD cannot form a complex with this probe (Fig. 8A, lane 6).

DISCUSSION

In this article, a novel approach for the isolation and identification of specific RNA substrates for a given RNA-binding protein (SNAAP) is described. This technique expands on existing approaches. It was used to isolate and identify specific mRNA substrates for the αCP1 RNA-binding protein. SNAAP has many advantages, one of which is that a cellular mRNA population and not random RNA is used as the source of RNA. In addition, the protein–RNA interaction occurs in a cell extract environment, where both mRNAs as well as additional proteins that might be necessary to form a specific complex are present. Therefore, both RNAs that bind to the RNA-binding protein of interest directly and RNAs that bind indirectly in a multiprotein complex (Fig. 1) can be isolated. In fact, we demonstrated that two of the mRNAs identified as interacting with αCP1 can actually be bound directly by the protein (Fig. 6). We were also able to show that the α-globin mRNA that is indirectly bound by αCP1 (Kiledjian et al., 1995; Wang et al., 1995) can also be isolated (Fig. 2). Additionally, the use of a second RNA-binding protein as a negative control allows for the elimination of nonspecific mRNAs that can interact with all RNA-binding proteins. This provides a means for eliminating false positives that are a common setback in this kind of interaction.

This study also demonstrated that the presence of protein in an extract is crucial in providing the specificity of the interaction between the substrate RNA and the RNA-binding protein under physiological salt conditions. The specificity observed when using extract rather than purified RNA was unexpected but informative. Our interpretation of these results is that the proteins that naturally coat an mRNA provide a competitive environment that restricts an RNA-binding protein to an interaction with its specific substrate(s). The mRNA in its natural mRNP state becomes less accessible to an exogenous RNA-binding protein. Only those mRNAs

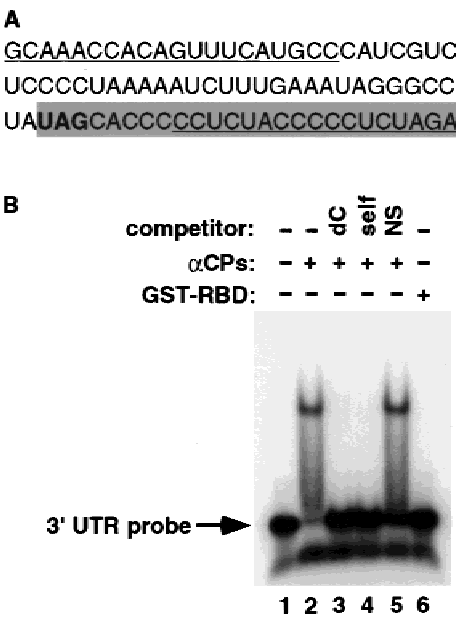

FIGURE 8. αCP binding site in the coxII 3′ UTR. **A**: The sequence of the terminal 116 nt of the coxII mRNA. The underlined sequences correspond to oligonucleotides used to construct the riboprobe used in previous EMSAs. The bold UAG is the stop codon. The shaded sequence represents the 3′ UTR region that was used in the EMSA in **B**. **B**: An EMSA using ^{32}P-5′ end labeled coxII 3′ UTR DNA oligonucleotide and purified αCP proteins. Lane 1 contains the probe alone. Two hundred nanograms of purified αCPs were added to the probe (lanes 2–5) and the complex formed was competed with 15 pmol of the following oligonucleotides: oligo dC (lane 3), a self oligonucleotide (lane 4) and a nonspecific oligonucleotide (lane 5). Two hundred and fifty nanograms of GST-RBD were added to the probe as a negative control (lane 6).

or mRNP complexes for which the fusion protein of interest has a high affinity at the physiological salt concentrations used would be bound and retained. Conversely, when purified RNA is used, the normal complement of cellular RNA-binding proteins is not present, so the test RNA-binding protein of interest can more readily associate with the mRNA. It is reasonable to assume that more stringent conditions might provide specificity to the purified RNA reactions. However, the physiological relevance of such conditions might be questionable. As shown in Figures 5–8, when using an EMSA assay under buffer conditions similar to those of the copurification assays, binding specificity is retained even though purified protein and nucleic acids are used. It is unclear why a dramatic difference in binding specificity is detected in the copurification assay but not in the gel-shift assay. Perhaps low-affinity interactions are more readily dissociated in the gel matrix of the EMSA assay, thus providing the observed specificity.

The large number of random 5′ primers in combination with any one of the three 3′ primers allows PCR amplification of any mRNA that has a poly(A) tail. It is important to realize that the cloned PCR product does not necessarily represent or include the binding site. The SNAAP technique allows for the identification of genes that are bound by the protein of interest. The exact binding site must be determined experimentally. Of interest is that the αCP binding sites in the TAPA-1 and coxII 3′ UTRs bear little homology to the existing α-complex consensus (Holcik & Liebhaber, 1997). Therefore, use of the SNAAP technique allows the identification of target RNAs that might contain an alternate binding sequence, even in cases where the binding site of an RNA-binding protein is known.

Another major advantage of this approach is its versatility. This technique is potentially applicable to any RNA-binding protein. The source of the extract as well as its nature can vary depending on the localization and function of the RNA-binding protein of interest. Here, K562 S130 extract was used as the source of mRNA and protein because αCP1-mediated stabilization of the α-globin mRNA is a cytoplasmic event (N. Day, M. Kiledjian, unpubl. observations). Whole-cell or nuclear extract can also be used, as well as extract from any type of primary or established cell line. Alternative approaches could also be envisioned, including expression of a fusion or epitope-tagged protein directly into cells, followed by protein–RNA complex isolation and RNA identification.

Using the SNAAP technique, novel target mRNAs for αCP1 including the TAPA-1 and coxII mRNAs have been identified. The TAPA-1 protein was originally described as the target antigen of monoclonal antibody 5A6, which inhibited growth of certain lymphoid cell lines (Oren et al., 1990). TAPA-1 is a component of the CD19/CD21/Leu-13/CD81 signal-transducing complex in B lymphocytes (Tedder et al., 1997). Although mice lacking TAPA-1 (Maecker & Levy, 1997; Miyazaki et al., 1997; Tsitsikov et al., 1997) undergo normal B- and T-cell maturation, CD19 expression and signaling in B cells is impaired and the number of B-1 cells is reduced (Miyazaki et al., 1997; Tsitsikov et al., 1997). Furthermore, TAPA-1 on the surface of B cells seems to be essential in promoting interleukin 4 secretion and antibody production during T helper type 2 immune responses (Maecker et al., 1998). Therefore, TAPA-1 plays an important role in CD19 expression and in B cell development and function.

The binding site of αCP to the TAPA-1 mRNA is contained within a 350-bp region encompassing nt 1130–1480 of the cDNA. This site was further localized to a 35-nt region of the 3′ UTR that includes nt 1331–1365 of the cDNA. As mentioned above, the binding site identified shows little homology to the existing α-complex consensus; none other than the fact that both sequences are C-rich. Although no data is currently available on the half-life of the TAPA-1 mRNA, the αCPs could be binding this message to stabilize it or binding could be influencing other posttranscriptional events including translation (Blyn et al., 1997; Gamarnik & Andino, 1997; Ostareck et al., 1997). The function of this interaction is currently under investigation.

The second target mRNA for αCP1 that was identified using the SNAAP technique was the coxII mRNA. The coxII gene is one of three mitochondrial DNA-encoded subunits of respiratory Complex IV, which is the final enzyme of the electron transport chain of mitochondrial oxidative phosphorylation. The coxII mRNA is transcribed as part of the H-strand polycistronic transcript of mitochondria. The tRNA(Asp) and tRNA(Lys) flanking the coxII mRNA are cleaved and the released mRNA is then polyadenylated (Attardi et al., 1982). This mRNA has a short 3′ UTR that contains two 9-nt direct repeats in tandem that are C-rich. These 25 nt are sufficient for direct binding by the αCPs. It is not known whether a single repeat is sufficient for binding. Interestingly, the binding site in coxII mRNA has little homology to the existing α-complex consensus. The differences in the binding site consensus sequence could be because TAPA-1 and coxII are bound directly by αCP1, whereas the α-globin mRNA is bound by a complex containing αCP1.

Two of the six transcripts that were identified as substrates for αCP1, the ND3 and the coxII genes, code for subunits of mitochondrial enzymes that are encoded in the mitochondrial DNA genome. Although αCP1 is present in the cytoplasm (Gamarnik & Andino, 1997; our unpubl. observations), it is not clear whether it is also contained within mitochondria. Perhaps the source of the ND3 and coxII mRNA is a nuclear transcript arising from fragmented mitochondrial DNA inserted into the nuclear genome as a result of mitochondrial damage due to aging, hydroxyl radicals, or autophagy (Shay & Werbin, 1992). Alternatively, αCP1 might localize within mitochondria. Further experiments are needed to address the function and relevance of αCP1 binding to coxII mRNA**.** As was the case with the ND3 and the coxII genes, the MnSOD gene codes for a mitochondrial enzyme but, in fact, this gene is encoded within the nucleus. Because the MnSOD mRNA is translated in the cytoplasm, it is, therefore, accessible to binding and regulation by the αCP1 protein.

The remaining two mRNAs that were identified as substrates for αCP1 are the L27a mRNA and palmitoylated erythrocyte membrane protein (MPP1) mRNA. The L27a gene codes for a large ribosomal subunit protein. In mammalian cells, ribosomal proteins and their mRNAs are abundant, and, most often, abundant proteins are encoded by stable mRNAs (Hargrove & Schmidt, 1989). Therefore, binding of αCP1 to the L27a mRNA could play a role in stabilizing this abundant mRNA. The MPP1 protein is an integral membrane protein and is the most extensively palmitoylated protein of the erythrocyte membrane. Its function remains unknown. MPP1 contains a SH3 (src homology 3) motif, found in several other proteins that associate with the cytoskeleton and are suspected to play important roles in signal transduction (Ruff et al., 1991).

The list of mRNAs presented here as interacting specifically with αCP1 is by no means an exhaustive one. Only eight out of 80 5′ random primers were used in the differential display reactions in this study. Furthermore, only PCR products that were reproducible were characterized to avoid false positives.

These data demonstrate the feasibility of the SNAAP technique in identifying and cloning RNAs specifically interacting with any RNA-binding protein. Numerous methods have successfully been used to identify RNAs bound by an RNA-binding protein. The SNAAP approach expands on these techniques and utilizes a more physiologically based system to identify specific mRNA targets and a convenient means to eliminate false positives. This technique could allow the identification of important RNA targets and shed light on the function of many RNA-binding proteins.

MATERIALS AND METHODS

Preparation of S130 extract

Isolation of the cytoplasmic S130 extract was as described (Kiledjian et al., 1997). Briefly, approximately 10^{10} human erythroleukemia K562 cells were collected, lysed in lysis buffer (2×10^8 cells/mL lysis buffer; 10 mM Tris-HCl, pH 7.5, 1 mM potassium acetate, 1.5 mM magnesium acetate, and 2 mM dithiothreitol [DTT]), and cell debris and nuclei were pelleted. The supernatant was layered over a 30% (w/v) sucrose cushion and centrifuged at 130,000 × *g* for 2.5 h. The S130 supernatant was collected without disturbing the S130-sucrose interface, glycerol was added to 5% (v/v), and it was stored at −70 °C.

SNAAP technique

Isolation of copurifying RNAs

The GST-αCP1 fusion protein was expressed in *Escherichia coli* BL21 as previously described (Kiledjian et al., 1999). The GST-RBD fusion protein was expressed from a construct created by cloning the *Eco*RI-*Sal*I RNA-binding domain of hnRNP U from pGEM4Δ5′Msc (Kiledjian & Dreyfuss, 1992) into the respective sites of the polylinker in the pGEX-4T-1 vector (Pharmacia Biotech). Cells expressing either fusion protein were resuspended in lysis buffer (20 mM HEPES, pH 7.6, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 2 μg/mL leupeptin, 0.5% aprotinin), disrupted by sonication, and insoluble matter removed with a 10-min spin at 31,000 × *g*. The extract was treated with 200 U/mL micrococcal nuclease (Pharmacia) to eliminate bacterial RNAs 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 ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA). Approximately 100 μg of GST-fusion protein was bound to 40 μL GST-beads in a total volume of 1 mL in RNA binding buffer (RBB; 10 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$, 150 mM KCl) 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 including the nucle-

ase was removed with ten 1-mL washes in RBB/0.5% TX and one 1-mL wash in RBB. The washed beads (which carry the fusion protein) were resuspended in 350 μL of RBB. Two hundred and fifty micrograms of cytoplasmic S130 extract that contains cytoplasmic proteins and mRNA was precleared with 20 μL of glutathione Sepharose beads to remove background RNAs that bind to the 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. Alternatively, 5 μg of S130 RNA were used instead of the 250 μg of S130 extract in a similar reaction. RNAs interacting nonspecifically with the fusion protein were 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% sodium dodecyl sulfate (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% EtOH. The dried RNA was resuspended in 10 μL DEPC-treated H_2O by heating to 65 °C for 5 min.

Identification of copurifying RNAs

Copurifying RNAs were subjected to the differential display technique (GenHunter Corp.). All of the copurifying RNAs isolated from a single GST interaction experiment described above were used in a reverse transcription reaction with one of three different 3′ primers containing a stretch of 11 T nucleotides followed by an A, C, or G nucleotide. The 250 μg of S130 extract used as the source of RNA contains ~5 μg of total RNA, of which only a small fraction (~0.02%) actually copurifies with the fusion protein. These primers anneal to the poly(A) tail of each mRNA. The first strand is then used in a PCR amplification reaction with the same 3′ primer and one of 80 random 5′ primers. The radioactively labeled PCR products are then resolved on PAGE to obtain a profile of the RNA population. The bands representing RNAs specifically interacting with αCP1 were excised from the gel, eluted by soaking in H_2O for 10 min and boiling for 15 min, reamplified, cloned into the PCR Trap vector (GenHunter Corp.), and sequenced. The sequence data was used to search GenBank.

PCR amplifications

The α-globin 3′ UTR was amplified using Taq polymerase (Promega) with the following buffer: 20 mM $(NH_4)_2SO_4$, 70 mM Tris-HCl, pH 8.8, 2 mM $MgCl_2$, 1 mM DTT, 1 mg/mL bovine serum albumin (BSA), 1% Triton X-100, 10% dimethyl sulfoxide (DMSO), and 2 mM of each dNTP and the following primers: 5′-GCTGGAGCCTCGGTAGCCGT-3′ and 5′-TTTTT GCCGCCCACTCAGACTTT-3′ for 35 cycles at 92 °C for 30 s, 54 °C for 45 s, and 72 °C for 40 s.

The γ-globin 3′ UTR was amplified using Taq polymerase (Promega) with the following buffer: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, and 2 mM of each dNTP and the following primers: 5′-AAGGTGCTGACTTCC TTGGG-3′ and 5′-ATCCTTGAAAGCTCTGCATC-3′ for 30 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s.

The TAPA-1 3′ UTR was amplified from reversed transcribed K562 total RNA using the same buffer as that of the α-globin 3′ UTR PCR but with the following primers: 5′-CTATAGGGGGTGGCGTGTATGAGTGGAG-3′ and 5′-AGC ATGCCTGATGTTCCTTC-3′ for 40 cycles at 94 °C for 30 s, 40 °C for 2 min, and 72 °C for 30 s. To generate the PCR product for the TAPA-1 riboprobe, a second PCR was done to incorporate the T7 promoter using the T7 primer (5′-CGTAATACGACTCACTATAGGG-3′) and the same 3′ primer used above for 5 cycles at 94 °C for 30 s, 25 °C for 2 min, and 72 °C for 30 s and 30 cycles at 94 °C for 30 s, 48 °C for 1 min, and 72 °C for 30 s. The RT-PCR reaction amplifying the TAPA-1 sequences used similar conditions except for the primers, which were replaced with the following: 5′-GCTCTGCCTGC TCAGCCAGG-3′ and 5′-GACGGAGTCAGGATGTTGTG-3′.

The coxII sequences were amplified using Taq polymerase (Promega) with the same buffer as that of the γ-globin 3′ UTR PCR but with the following primers: 5′-CGTAATACGA CTCACTATAGGGGCAAACCACAGTTTCATGCC-3′ and 5′-GGCTCTAGAGGGGGTAGAGG-3′ for 2 cycles at 94 °C for 1 min, 54 °C for 45 s, 72 °C for 40 s and 23 cycles at 94 °C for 30 s, 54 °C for 45 s, 72 °C for 40 s. This PCR incorporates the T7 promoter at the 5′ end of the PCR product so it can be in vitro transcribed.

The GAPDH sequences were amplified using Taq polymerase (Promega) with the same buffer as that of the γ-globin 3′ UTR PCR but with the following primers: 5′-TGGACTGTGG TCATGAGTCC-3′ and 5′-ACCATGGAGAAGGCTGGGGC 3′ using the same cycling conditions as the coxII PCR above.

Riboprobe generation

Uniformly ^{32}P-labeled riboprobe was generated using approximately 400 ng of a PCR product containing the T7 promoter as template in 1× transcription buffer (40 mM Tris-HCl, pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine, and 0.05% Tween-20) and 5 mM each of rATP, rCTP, GTP, and 3 mM UTP along with T7 Polymerase (Promega) and ^{32}P-rUTP. After 1 h, RQ1 DNase (Promega) was added and incubated for an additional 15 min. Unincorporated nucleotides were removed using a G-50 column (Pharmacia Biotech).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays were carried out with approximately 0.5 ng of in vitro transcribed [^{32}P]-UTP uniformly labeled TAPA-1 or coxII RNAs (~10,000 cpm) per reaction. Binding reactions were carried out with S130 extract pretreated for 15 min on ice with 1 U ACE-RNase inhibitor (5′-3′) per 30 μL S130 extract. Reactions were carried out in RBB containing heparin at 5 mg/mL and 300 ng of poly(U) competitor RNA to minimize nonspecific RNA–protein interactions and were incubated for 20 min at room temperature with 40 μg of S130 extract in a total volume of 15 μL. Specific competitor was added at the beginning of the reaction where indicated. The specific oligo dC competitor is a 16-base oligonucleotide containing only C residues. Nonspecific oligonucleotide competition was done using a 16-base oligonucleotide containing random nucleotides flanking four C residues. This oligonucleotide was shown not to compete the αCPs (Kiledjian et al., 1999). A stretch of nine Cs in a row is needed for efficient competition. The complexes were resolved on a 5% polyacrylamide gel (60:1 acrylamide:bis) in 0.5× TBE buffer at 8 V/cm (Kiledjian et al., 1995).

Purification of the αCP proteins

The poly(C)-binding αCP proteins were purified from 10^{10} K562 cell cytosolic extract to apparent homogeneity using DEAE Sephacel, SP-Sepharose and ssDNA chromatography as previously described (Kiledjian et al., 1995, 1999).

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