Heterogeneous nuclear ribonucleoprotein K (hnRNP K) protein associates with multiple mitochondrial transcripts within the organelle.

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Heterogeneous nuclear ribonucleoprotein K (hnRNP K) protein interacts with a subset of cellular RNAs. We used K protein as a bait in the yeast three-hybrid screen to identify RNAs that bind K protein in vivo. A large number of K protein-binding RNA clones were identified from a human hybrid RNA library. These sequences consisted of C-rich patches and were G-poor. Unexpectedly, several of the RNA clones were encoded by the mitochondrial genome. In a subsequent three-hybrid screen of a hybrid RNA library generated from a mouse liver mitochondrial genome, K protein bound RNA sequences encoded by different loci spanning nearly the entire mitochondrial genome. Western blot analysis of extracts from mitochondria and mitochondrial fractions showed that K protein is localized within mitoplasts. Reverse transcriptase PCR of RNA co-immunoprecipitated with K protein from lysates of isolated mitochondria showed that K protein is associated with several processed mitochondrial transcripts. In contrast, in the same assay, the polycistronic nascent mRNA bound K protein weakly or not at all. Results of this study suggest that K protein acts within functional modules that are responsible for expression of genes in mitochondria.

EXPERIMENTAL PROCEDURES

Materials—The yeast three-hybrid system pOAA and pHIMS2-2 plasmids and Saccharomyces cerevisiae L40 strain were a gift from Dr. Stanley Fields (Department of Medicine, University of Washington, Seattle, WA) (25).

Construction of Hybrid RNA Libraries for the Yeast Three-hybrid Screens—To construct human hybrid RNA libraries, total cellular RNA was isolated from human gastric carcinoma AGS cells using TRIzol reagent (Invitrogen) following the manufacturer's protocol. Random hexamer-primed cDNAs were synthesized using the cDNA synthesis system (Invitrogen). Double-stranded cDNA was digested with AluI, HaeIII, PvuII, and SspI and then fractionated on 2% agarose gel. Fragments ranging from 50 to 150 bp were purified from the gel and ligated to Smal-digested and dephosphorylated pHIMS2-2 plasmid. To construct a mouse mtRNA library, mitochondria isolated from mouse livers were used to purify mtDNA. mtDNA was cut with AluI, HaeIII, and SspI, and 50–150-bp fragments were purified and ligated into Smal-digested and dephosphorylated pHIMS2-2 plasmid. The ligation mixture was used to transform competent DH5α cells.

Screening of RNA Libraries—The K protein-activation domain hybrid was constructed by insertion of the full-length K protein cDNA (18) into the pOAA plasmid. Yeast L40 cells were transformed with the K protein hybrid and the library plasmids using the LiAc/polyethylene glycol method (26). Cells were grown on synthetic medium lacking leucine, histidine, uracil, tryptophan, and adenine. Colonies were picked after 4–5 days and plated onto fresh selective plates containing 1 J. Ostrowski and K. Bomsztyk, unpublished data.

2 The abbreviations used are: KH, K homology; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; RT, reverse transcriptase.
2.5 mM 3-aminotriazole; and when the cells had grown, β-galactosidase activity was tested by X-gal filter assay. To eliminate false positives, the K protein hybrid plasmid was purged from the colonies by growing cells in liquid medium without uracil but containing 2× concentration of adenine and leucine. Cells without the K protein-activation domain construct were used as controls for β-galactosidase activity. The positive plasmids were isolated from yeast cells, and DNA inserts were sequenced.

To test the K protein-binding affinity of the isolated RNA clones, hybrid RNA-expressing plasmids were re-transformed into L40 cells containing the pOAA-K protein plasmid, and the transformants were tested again by X-gal filter assay. Selected yeast clones were grown in liquid culture until mid-log phase (A600 = 0.5–0.6). Cells were lysed, and direct quantitative measurements of β-galactosidase activity assays were carried out as previously described (9). Enzymatic assays represent the average of at least three independent colonies for each hybrid RNA-expressing plasmid.

**Computational Analysis of RNA Sequences**—The sequences of all RNA fragments were identified using blastn.3 Similarity searches were conducted in the nr data base (non-redundant data base of sequences deposited in the DDBJ/GenBank™/EMBL Data Bank and the Protein Data Bank) and in the dbEST data base (expressed sequence tags in the DDBJ/GenBank™/EBI Data Bank) to identify the RNA. RNA secondary structures were predicted using the mfold Version 3.1 server (27). The ClustalW program (28) was used for multiple alignment of the RNA fragments. Graphs were generated using Excel programs (Microsoft). Statistical analysis was conducted using Statistica PL.

**Isolation of Mitochondria**—Anesthetized mice were killed, and the livers were resected, cut into small pieces with scissors, and homogenized in a Potter-Elvehjem (Teflon-glass) homogenizer with 10 volumes of cold homogenization buffer (225 mM mannitol, 75 mM sucrose, 0.5 mM EGTA, 10 mM Tris-HCl (pH 7.4), 10 µg/ml leupeptin, 0.5 mM diithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 650 × g for 10 min, and the supernatant containing the post-nuclear fraction was centrifuged again at 650 × g for 10 min. The post-nuclear supernatant was centrifuged at 11,000 × g for 10 min in a swing-out rotor. The mitochondrial pellet was resuspended carefully in homogenization buffer and centrifuged again at 11,000 × g for 10 min. The crude mitochondrial fraction suspended in an isotonic sucrose buffer (0.25 M sucrose, 1 mM EGTA, 10 mM Tris-HCl (pH 7.4), 0.5 mM diithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10 µg/ml leupeptin) was layered on a 1.0/1.5 M discontinuous sucrose gradient and then centrifuged at 80,000 × g for 1 h. Mitochondria were collected from the phase between 1.5 and 1.0 M sucrose and washed several times with the same sucrose buffer.

**Subfractionation of Mitochondria**—Mouse mitochondria were subfractionated as previously described (29). Briefly, mitochondria suspended in an isotonic sucrose buffer (0.25 M sucrose, 1 mM EGTA, 10 mM Tris-HCl (pH 7.4), 0.5 mM diithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10 µg/ml leupeptin) were sonicated and then centrifuged at 320,000 × g for 1 h at 4 °C. The soluble mitochondrial fraction was recovered from the supernatant. The mitochondrial membrane fraction was obtained by homogenization of the pellet in immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EGTA, 1% Triton X-100, and 0.5% Nonidet P-40).

To obtain mitoplasts (29), freshly prepared mitochondria were diluted 10-fold with hypotonic buffer (5 mM Tris-HCl (pH 7.4) and 1 mM EDTA) and centrifuged at 14,000 × g for 20 min at 4 °C. The pellet was washed four times with hypotonic buffer, and proteins from mitoplasts were extracted using immunoprecipitation buffer. The protein concentration was measured using the protein assay (Pierce), and K protein content in mitochondrial protein fractions was analyzed by Western blotting.

**Western Blotting**—Equal amounts of sample protein (100 µg) were boiled in 2× loading buffer (125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 10% β-mercaptoethanol) for 5 min. Proteins were separated by 10% SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 for 1 h at room temperature. The membranes were then probed for 1 h at room temperature with rabbit anti-K protein antibodies. After washing, the membranes were incubated for 60 min at room temperature with secondary antibodies conjugated with alkaline phosphatase, and immunoreactions were detected using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium phosphatase substrate.

**Isolation of Total Cellular and Mitochondrial RNAs**—Total cellular RNA was prepared as previously described (17). Total mtRNA was prepared from mitochondria that were isolated from livers of anesthetized mice using TRIzol reagent following the manufacturer’s protocol. RNAse inhibitor (0.1 unit/µl) was used in all procedures involving RNA.

**Co-immunoprecipitation of RNA with K Protein**—Frozen hepatic tissue or isolated mitochondria were pulverized under liquid nitrogen, and the powders were homogenized using immunoprecipitation buffer. In addition to diithiothreitol (0.5 mM), phenylmethylsulfonyl fluoride (0.5 mM), and leupeptin (10 µg/ml), the lysis buffer contained the following phosphatase inhibitors: 30 mM p-nitrophenyl phosphate, 10 mM sodium fluoride, 0.1 mM sodium orthovanadate, 0.1 mM sodium molybdate, and 10 µg/ml glycerophosphate.

To co-immunoprecipitate RNA with K protein, we used a previously described procedure (17). Briefly, hepatic or mitochondrial lysates were first precleared with 50 µg of rabbit IgG (Bio-Rad) by bath-sonicating for 15 min at 4 °C, followed by binding to 20 µl of protein A beads for 45 min at 4 °C. Beads were spun down, and the supernatant was bath-sonicated with 20 µl of affinity-purified anti-K protein antibody 54 for 15 min at 4 °C. The complexes were pulled down by adding protein A beads (20 µl) and rotating the slurry for 45 min at 4 °C. Beads were washed four times with 1 ml of immunoprecipitation buffer, and RNA was eluted from the beads with 100 µl NaCl and 1% SDS at 65 °C for 10 min in an Eppendorf Thermomixer. RNA was phenol/chloroform-deproteinized and then ethanol-precipitated. RNA pellets were resuspended in water and stored at −70 °C.

**RT-PCR**—RT reactions were carried out using SuperScript II RT (Invitrogen) and random primers in a 20-µl volume following the manufacturer’s protocol. RT reactions were diluted 1:10 with water, and cDNAs were used in PCR.

**RESULTS**

Identification of K Protein-binding RNAs Using the Yeast Three-hybrid Screen—To identify the spectrum of K protein-binding RNAs, we used full-length K protein as a bait in the yeast three-hybrid screen (25). In this screen, protein–RNA interaction is detected by the reconstitution of a transcriptional activator using two-hybrid proteins and RNA from a hybrid RNA library. The hybrid RNA molecule is recruited to the promoter of a reporter gene by binding to a hybrid protein consisting of the bacteriophage MS2 coat protein fused to the DNA-binding protein LexA. The hybrid RNA, in turn, recruits the hybrid RNA-binding protein of interest. The hybrid RNA consists of binding sites for the MS2 coat protein and the RNA sequence that binds the protein of interest. The RNA-binding protein is generated by fusing it to the transcriptional activation domain of the yeast Gal4 protein (25, 30).

Transformants were selected on yeast synthetic medium lacking leucine, histidine, uracil, tryptophan, and adenine. The His-deficient medium selected clones that expressed the HIS3 reporter gene driven by LexA DNA-binding elements. These clones were then tested by β-galactosidase assay for lacZ reporter gene expression also driven by LexA DNA-binding sites. The putative positive clones that failed to activate the lacZ reporter gene in the L40 cells in the absence of the pOAA-K protein plasmid were considered to be true positives.

Five to ten million independent human RNA clones generated from total cellular RNA were screened. Forty-three true positive clones were identified. Unexpectedly, several positive clones represented mRNAs (see Fig. 2B). Sequencing of 20 randomly chosen plasmids from the hybrid cDNA library revealed no mtRNA sequences. This suggests that the cloning of mtRNA by K protein in the yeast three-hybrid system does not reflect overrepresentation of mitochondrial sequences in the cDNA library. Instead, these results suggest preferential K protein binding to mitochondrial transcripts in the yeast three-hybrid system.

**Computational Analysis of RNA Sequences Cloned in the Yeast Three-hybrid Screen**—Computer-generated predictions
of secondary structures of the RNAs (mfold) were used to determine any similar secondary structure among positive clones. The temperature was set at 37 °C, and 1 M NaCl was used during the calculation (27, 31). This computer-based analysis did not identify shared secondary structure among the isolated RNA clones.

To define the consensus K protein-binding motif, the primary RNA sequences of the positive clones were compared using the ClustalW program (28) for multiple alignment of the RNA fragments. All positive clones exhibited a strong blue color in the presence of X-gal. We used liquid assay for β-galactosidase activity to estimate the efficiency of interactions. The β-galactosidase activities ranged between 0.5 and 663 units and were compared with the results of alignments. A preliminary alignment was conducted only with those RNA sequences that exhibited β-galactosidase activity 25 units and above. The remaining sequences were added to the alignment manually using the BIOEdit program. Analysis of the alignment revealed a tandem array of three C-rich patches with the following sequence: (C/U)(C/U)AUCN2-C(U/C)AACC(C/A)N1-1,UCA(C/U).

The frequency of ribonucleotides at all positions of the 50-base alignment is shown in Fig. 1. The three C-rich patches (left, middle, and right) are shown. The high frequency of the single A and U in the left and right patches is easily seen. The frequency of a single A and U in the middle patch is lower. Mean cytosine content of the aligned regions was significantly higher than the mean cytosine content of the clones as a whole (p < 0.05). Cytosine content was 39.8%, adenine 25.4%, uracil 24.4%, and guanine 10.3% of the total. Thus, the overall K protein-binding RNAs are C-rich and G-poor. This is consistent with the observation that K protein strongly binds poly(C) RNA, but not poly(G) RNA (17, 32). There was a strong correlation between K protein-binding affinities of the cloned RNAs and the number of sequence regions containing the three C-rich boxes of the predicted binding motif (Table I). This is not unexpected because RNA clones containing multiple copies of the consensus sequence would be expected to recruit multiple K protein molecules, resulting in proportionally stronger activation of the lacZ reporter gene.

To test the utility of the computationally derived K protein-binding motif, we carried out analysis of the 15-lipoxygenase (33) and human papilloma virus type 16 L2 (16) mRNAs, two transcripts known to be regulated by K protein. The rabbit 15-lipoxygenase transcript contains three C-rich stretches in the regulatory region that agree well with the derived consensus sequence (Table II). The human papilloma virus type 16 L2 mRNA contains five such tandem sequences (Table III). Three of these elements are located within the region known to be important in translational regulation of this viral transcript (16).
 precipitated K protein with associated RNAs from both whole cell and mitochondrial lysates (Fig. 3B). RT reactions were carried out with total mtRNA (Fig. 3B, lane 2), mtRNA precipitated with K protein (lane 3), and RNA precipitated with K protein from total cell lysates (lane 4). PCR using primers to actin cDNA revealed the predicted size product in RT from RNA immunoprecipitated with K protein from total cell lysates (Fig. 3B, lane 4), but not in RT from RNA from total mitochondrial lysates (lane 2) or in RT from RNA co-immunoprecipitated with K protein from mitochondria (lane 3). This result provides

TABLE I
Sequences of the RNA clones isolated from the yeast three-hybrid system that exhibited the highest β-galactosidase (β-gal) activity of the lacZ reporter gene

| Insert code | β-gal activity (units) | Derived binding motif |
|-------------|------------------------|-----------------------|
| E8          | 662                    | CUGAUGCCCAUGAGG       |
| B17         | 373                    | CGUGAUGCCCAUGAGG      |
| ins.6       | 248                    | AACUGAUGCCCAUGAGG     |
| B6          | 256                    | UCCUGAUGCCCAUGAGG     |
| B10         | 177                    | UAUAUGCCCAUGAGG       |
| C7          | 169                    | AUAGAUGCCCAUGAGG      |
| C13         | 108                    | AAUGAUGCCCAUGAGG      |
| C3          | 88                     | GAUGAUGCCCAUGAGG      |
| G4          | 70                     | GAGAUGCCCAUGAGG       |
| F6          | 64                     | GUGAUGCCCAUGAGG       |
| D14         | 61                     | CAGAUGCCCAUGAGG       |
| C14         | 57                     | AGAUGCCCAUGAGG        |
| E20         | 52                     | CGAUGCCCAUGAGG        |
| F5          | 50                     | UGAUGCCCAUGAGG        |
| MIU         | 46                     | UGAUGCCCAUGAGG        |
| E18         | 41                     | AGAUGCCCAUGAGG        |
| C4          | 29                     | CGAUGCCCAUGAGG        |
| F2          | 26                     | UGAUGCCCAUGAGG        |
| G13         | 16                     | AGAUGCCCAUGAGG        |
| G10         | 16                     | CGAUGCCCAUGAGG        |
| B1          | 13                     | AAUUGCCCAUGAGG        |
| G16         | 10                     | UGAUGCCCAUGAGG        |
| E10         | 8                      | AGAUGCCCAUGAGG        |
| G8          | 8                      | UGAUGCCCAUGAGG        |

TABLE II
Sequence of the 3'-untranslated rabbit reticulocyte (RBC) 15-lipoxygenase (15-LOX) mRNA regulatory region

M27214 Oryctolagus cuniculus (rabbit) RBC 15-LOX mRNA.

\[210^{3}\text{agccccucgccuuucccuaagccucucuuucccccagccccgccuccuccc}a\text{c} \]
further proof that the mitochondrial fraction was not contaminated with cytosol. Within mitoplasts, proteins involved in RNA-directed processes can be associated with the inner membrane, the soluble fractions, or both (38). We used anti-K protein antibody in Western blotting to assay for the presence of K protein in these submitochondrial fractions. Western blotting revealed that K protein copurified with the membrane fraction (Fig. 3A, lane 4), but was not found in the soluble fraction (lane 3). These results suggest that K protein is associated with the mitochondrial inner membrane.

**K Protein Coprecipitates Processed Mitochondrial Transcripts**—The yeast three-hybrid screen showed that K protein bound many RNA sequences encoded by the mitochondrial genome (Fig. 2). We next used RNA immunoprecipitation assay to test whether K protein associates with mitochondrial transcripts in vivo. Mitochondria were isolated, and lysates were prepared using immunoprecipitation buffer. After immunoprecipitation with anti-K protein antibody, eluted RNA was used as a template in the RT reaction (Fig. 4). As a control, total mtRNA was used in the RT reaction. Pairs of oligonucleotide primers covering loci encoding several mitochondrial enzymes were used in PCR. PCR fragments of the predicted size were obtained for sequences corresponding to ATP synthase subunit 6, NADH dehydrogenase subunits 4 and 5, and cytochrome b with either mtDNA template (Fig. 4B, lane 2) or RT template from either total (lane 3) or immunoprecipitated (lane 4) RNA.

**TABLE III**

| Offset in genome | Sequence |
|------------------|----------|
| 4266             | acuguacauccgucuccacuuuaaguauuauaaaagcucauaaa |
| 4701             | cuacuaagaccucacauccacauccacauccacauuaaauuuu |
| 4780             | acuacuacauccaucuauuuauuucacgucucugucucuauuaa |
| 5107             | gcaaccauaaccatgucacugacucagucacagucaguggu |
| 5377             | gcaaccaauacagcauacacacauacacacauacacacaguaua |

**Fig. 2.** Position within mitochondrial genome of the human and mouse RNA clones identified in the yeast three-hybrid screen using K protein as the bait. A, list of the 13 proteins and two rRNAs encoded by the human and mouse mitochondrial genes. B and C, arrangement of human and mouse mitochondrial (mt) genes transcribed from the heavy strand is shown by the white boxes, and the protein transcribed from the light strand is shown by the hatched boxes. The rulers below represent the entire human (16,568 bp; NCBI accession number NC_001807) and mouse (16,295 bp; NCBI accession number NC001569) mitochondrial genomes. The positions of the arrowheads corresponds to the 5’-ends of the isolated clones.

**Fig. 3.** Intramitochondrial localization of K protein. A, mitochondria were isolated from livers of anesthetized mice, and whole mitochondria and mitochondrial fractions were prepared as described under “Experimental Procedures.” Proteins from whole mitochondria (lane 1), mitoplasts (lane 2), soluble mitochondrial fractions (lane 3), and mitochondrial membrane fractions (lane 4) were separated by SDS-PAGE and immunostained (IS) on polyvinylidene difluoride membrane using anti-K protein antibody (aK). B, RT reaction was performed using random primer on total mitochondrial (mt) RNA (lane 2), mtRNA immunoprecipitated (IP) with K protein (lane 3), and RNA immunoprecipitated with K protein from total cell lysates (lane 4). PCR was done using primers to actin cDNA. After agarose gel electrophoresis, PCR products were visualized by ethidium bromide. A DNA ladder (Gene-Ruler, 100 bp, MBI Fermentas) is shown in lane 1.
Fig. 4. K protein co-immunoprecipitates several protein-coding mitochondrial transcripts. A, arrangement of mouse mitochondrial protein-coding genes is shown as in Fig. 2C. The pair of arrows designates the location of the pair of primers used in PCR that is shown in the adjacent gel stained with ethidium bromide shown in B. The distance between the arrowheads corresponds to the size of the predicted PCR product. The ruler below represents the entire mouse mitochondrial genome in kb. B, mitochondria isolated from mouse livers were used to prepare mitochondrial (mt) DNA, total RNA, and RNA immunoprecipitated (IP) with K protein (see "Experimental Procedures"). Total mtRNA and RNA pulled down with K protein were used in the RT reaction with random primers. PCRs (30 cycles) were carried out with the following primer pairs: mt.1S (CTCCTAGGCTTTTACACCACA) and mt.1AS (GAGGGTGAATACGTTAGGCGTGA), mt.3S (ATGATGTACGTTACACCA) and mt.3AS (AATGTTTGTTGTACCATC) and mt.6S (GGAGACCAGACACTACATCC) and mt.6AS (ATATAGTTGTAGTTGAG), and mt.2S (AGGGACACTTATATTGAG) and mt.2AS (GAGGGTGAATACGTTAGGCGTGA). The templates used were mtDNA (lane 2) and RT from total (lane 3) and immunoprecipitated (lane 4) RNAs. Total RNA (lane 5) and immunoprecipitated RNA (lane 6) are shown without RT. After agarose gel electrophoresis, PCR products were visualized by ethidium bromide. A DNA ladder (GeneRuler, 100 bp) is shown in lane 1. ND, NADH dehydrogenase.

Reaction without RT yielded no PCR products (lanes 5 and 6), indicating no DNA contamination. The results of RT-PCR analysis suggest that K protein binds these mitochondrial protein-encoding transcripts in vivo. The NADH dehydrogenase 4L and 4 subunits are encoded by a bicistronic transcript (34). Using a set of primers encompassing sequences that encode portions of both enzymes yielded the predicted size PCR fragment, suggesting that K protein binds in vivo this bicistronic mRNA as well.

The mitochondrial genome is transcribed from both the heavy and light strands of the circular DNA, generating two long polycistrionic RNAs. Nearly every protein gene and the two rRNA genes are flanked by tRNA-encoding sequences. The precise endonucleolytic excision of tRNAs from the polycistrionic transcript is thought to generate not only the correct ribonucleoprotein E, which like K protein, contains three KH domains and binds a consensus sequence consisting of three tandem C-rich patches. This result is not unexpected because the K protein KH domains bind C-rich sequences (40). The fact that this consensus sequence contains three tandem short C-rich domains may mean that the strong binding of these RNAs is mediated by simultaneous three-way KH domain-C cluster interactions. A similar mode of binding has been suggested for heterogeneous nuclear ribonucleoprotein E, which like K protein, contains three KH domains and binds a consensus sequence consisting of three tandem C-rich patches (41).

Using the SELEX method, Thisted et al. (41) identified a short K protein-binding RNA sequence consisting of a single C-rich cluster, UC₃₋₄(U/A)(U/A). Although this empirically derived sequence is also C-rich, a single rather than tandem motif was sufficient to mediate strong protein binding. The short length of this motif suggests a single KH domain engagement. There are several possibilities to explain the apparent discrepancy between the consensus sequence derived in this study and the sequence obtained through SELEX (41). (i) The SELEX method uses sequences that are only 20 bases long and therefore cannot detect long RNA-binding sequences. (ii) The yeast three-hybrid screen might pick up only the strongest binding RNAs. (iii) The yeast three-hybrid screen is an in vivo approach, whereas the SELEX method is an in vitro strategy. We have previously shown that nearly the entire cellular repertoire of deproteinized mRNA binds K protein in vitro (3). In contrast, only a subset of mRNAs bind to K protein in vivo (17). This suggests that, in vivo, the structure of RNA is not depend-
From total RNA (lanes 4, 8-12), IP immunoprecipitated (lanes 3) with K protein (lanes 3). PCR products were visualized by ethidium bromide. A DNA ladder is shown in lane 2. mt were mitochondrial (mt) DNA (mt.6AS (ATAAATGGGTGTTCTACTGGTTGG). The templates used in PCR (lanes 3, 7, and 11), and RT from total RNA (lanes 4, 8, and 12). After agarose gel electrophoresis, PCR products were visualized by ethidium bromide. A DNA ladder is shown in lane 1.

**FIG. 5.** K protein does not precipitate the unprocessed polycistronic mitochondrial mRNA. A, arrangement of mouse mitochondrial protein-coding genes is shown as in Fig. 2C. The pair of arrows designate the location of the pair of primers (I, II, and III) used in PCR shown in B. The distance between the arrowheads corresponds to the size of the predicted PCR product. The ruler below represents the entire mouse mitochondrial genome in kb. B, PCRs (30 cycles) were carried out with following pairs of primers: mt.1S (CTCCTAGGCTTTACCATACA) and mt.2AS (GTGTAAGCGTTGATTTGTTGTC), mt.3S (TAGCATACCCCTCATCTTC) and mt.4AS (GTCTGTTCGTCCGTCACATAC), and mt.5S (GATTCCACCCCCTCACGACTAA) and mt.6AS (ATAAATGGGTGTTCTACTGGTTGG). The templates used were mitochondrial (mt) DNA (lanes 2, 6, and 10), RT from RNA immunoprecipitated (IP) with K protein (lanes 3, 7, and 11), and RT from total RNA (lanes 4, 8, and 12). After agarose gel electrophoresis, PCR products were visualized by ethidium bromide. A DNA ladder is shown in lane 1.

ent only on nucleic acid sequences, but also on other determinants, including proteins that may compose the target ribonucleoprotein complex. (iv) The apparent discrepancy between SELEX and the yeast three-hybrid screen may simply reflect the fact that there are different classes of K protein RNA targets. The class of RNA targets may be defined by the number and/or the combination of the three KH domains that participate in the binding. For example, there may be a class of RNA targets that engage only one or two of the three KH domains, e.g. concurrent RNA binding involving the KH1 and KH3 domains or just RNA binding of a single KH domain. Because the KH3 domain is sufficient to mediate strong K protein-poly(C) binding in vitro (42), engagement of a single KH domain is a plausible explanation for the SELEX-derived sequence (41).

The three-hybrid screen identified many mtRNAs that bound K protein in the yeast system (Fig. 2). This finding does not reflect overrepresentation of mitochondrial sequences in the hybrid library, suggesting preferential K protein binding of mtRNAs in this system. There are at least two possible explanations for this observation. First, of the identified human and mouse mtRNAs, all are C-rich and G-poor and contain >30 copies of the consensus region consisting of the three tandem clusters. Second, as alluded to earlier, the structure of RNA in vivo is governed not only by nucleic acid sequence, but also by other determinants in the in vivo microenvironment such as ionic strength, temperature, and bound proteins that may favor selection of mitochondrial clones. That determinants other than the sequence itself play a key role in K protein-RNA interactions is underscored by the finding that the polycistronic nascent mitochondrial transcripts did not co-immunoprecipitate with K protein (Fig. 5), whereas several of the processed transcripts did (Fig. 4). Regardless of the reason why K protein preferentially binds mtRNA in the yeast three-hybrid screen, this fortuitous observation allowed us to uncover a novel intramitochondrial interaction of a nuclear encoded protein with several mitochondrial transcripts (Figs. 2 and 3).

Mitochondrial protein import is mediated by a complex network of protein translocases in the outer and inner membranes along with a host of chaperones and processing enzymes found in the intermembrane space and in the mitochondrial matrix (43). TOM (translocase of the outer membrane) and TIM (translocase of the inner membrane) mediate both the translocation and the sorting of proteins to the outer membrane, intermembrane space, inner membrane, and mitochondrial matrix. The mitochondrial proteins contain targeting and sorting information consisting of defined amino acid sequences and/or other physicochemical determinants that are recognized by the TOM and TIM machinery (43). The classical mitochondrial targeting and sorting signals consist of N-terminal positively charged, hydrophobic, and hydroxylated amino acids that can form an amphipathic α-helix (44). It has become recently apparent that the mitochondrial targeting and sorting information is more diverse in that the amphipathic α-helix can be present not only in the N-terminal presequence, but also at an internal position. Moreover, there can be multiple internal targeting signals of yet to be characterized motifs (45, 45).

Computer-based analysis of K protein amino acid sequence did not identify motifs that form structures resembling known mitochondrial localization signals (TargetP Version 1.01) (46, 46). This poses a question of how a fraction of K protein is recognized and directed to the inner membrane by mitochondrial import machinery. K protein, by itself or in a complex with a chaperone, is likely recognized and translocated into intermembrane space by the TOM complex of the outer membrane. In yeast, sorting of protein into the inner membrane is carried out by the TIM22 complex, whereas translocation across it is mediated by the TIM23 complex (43). Thus, once delivered into the intermembrane space, with or without a chaperone, K protein could be recognized by the mammalian machinery similar to the TIM22 complex that would direct it to the matrix side of the inner membrane. Alternatively, and perhaps more likely, K protein could be translocated into the matrix via the mammalian TIM23 system and then could be recruited to the inner membrane by a membrane-anchored protein complex such as Oxa1 (47).

What could be the role of K protein in the mitochondrial processes? One emerging general model of K protein action is that of a transducer linking signal transduction pathways to sites of nucleic acid-directed processes (1, 3, 17, 21). Several studies have shown that K protein regulates mRNA translation (10, 16, 33). With respect to K protein-RNA interaction, K protein may play a role in mitochondrial expression of mitochondrial genes is regulated by extracellular signals, including ligands such as thyroid hormone (36) and insulin (48). In this regard, insulin alters K protein interactions with RNA (17). These studies suggest that K protein could be involved in the transmission of insulin and other signals to target genes and/or transcripts within mitochondria.

Proteome analysis of Jurkat T cells identified 37 proteins modified during apoptosis, including K protein (13). This suggests that K protein might be involved in the apoptotic pro
cesses as well. Because mitochondria play a crucial role in the execution of cell death (49), mitochondrial K protein may participate in this process. During phorbol 12-myristate 13-acetate-induced apoptosis, protein kinase Cδ translocates to the mitochondria (50). Protein kinase Cδ phosphorylates and binds K protein (2). These observations raise the following questions: does the K protein-protein kinase Cδ interaction play a role within mitochondria during apoptosis, and is this interaction functionally related to the binding of K protein to mitochondrial transcripts?

In conclusion, we have shown that K protein bound several mitochondrial transcripts in the yeast three-hybrid screen. In this system, strong binding of K protein to its RNA targets was mediated by regions consisting of three tandem C-rich patches. K protein was found within mitochondria and precipitated several mitochondrial transcripts. These results suggest that K protein acts as a transducer of signals within the functional modules that compose gene expression within mitochondria.

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