A novel principle for conferring selectivity to poly(A)-binding proteins: interdependence of two ATP synthase β -subunit mRNA-binding proteins

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Based on electrophoretic mobility-shift assays and UV cross-linking experiments, we present evidence in the present work for the existence of two mammalian cytosolic proteins that selectively interact with the 3'-untranslated region of the mRNA coding for the catalytic β -subunit of mitochondrial ATP synthase (β -mtATPase). One of the proteins, β -mtATPase mRNA-binding protein (BARB)1, is a novel poly(A)-binding protein that specifically binds the poly(A) tail of the β -mtATPase transcript. BARB1 achieves this mRNA selectivity through its interaction with a second protein, BARB2, that binds the β -mtATPase mRNA through a 22-bp element with a uridylate core, located 75 bp upstream of the poly(A) tail. Conversely, in the absence of

BARB1, BARB2 is still able to bind the β -mtATPase mRNA, but does so with lower affinity. Thus the interaction between BARB1 and BARB2 and β -mtATPase mRNA involves the formation of a complex between the two BARB proteins. We conclude that BARB1 and BARB2 selectively bind the 3'-untranslated region of β -mtATPase mRNA in a novel and interdependent manner. The complex between these two proteins may be involved in post-transcriptional regulation of gene expression.

Key words: β -subunit of mitochondrial ATPase, electrophoretic mobility-shift assay, mitochondrial ATP synthase biosynthesis.

INTRODUCTION

Mitochondrial ATP synthase (mtATPase) is composed of 16 subunits encoded by both the nuclear and the mitochondrial genomes [1]. To meet with different cell and tissue demands for respiratory energy, the cell would thus be expected to control the expression of all the corresponding genes in parallel [2]. This does not, however, seem to be the case. For example, in brown adipose tissue (BAT), the levels of all mtATPase mRNAs, with the exception of subunit c, are as high as in the mtATPase-rich heart, despite the fact that BAT has only one-tenth the amount of mtATPase [3,4]; the final level of the mtATPase is therefore apparently determined only by the level of subunit c mRNA, and all other mtATPase subunit mRNAs are found in considerable excess. Thus the translation of these highly expressed mtATPase mRNAs in BAT appears to be controlled by mechanisms that operate after the establishment of steady-state mRNA levels [5]. Similarly, a discrepancy between the mRNA levels coding for the catalytic β -subunit of mtATPase (β -mtATPase) and protein levels of the enzyme has been observed in a number of other rat tissues [6]. These findings are clearly indicative of the involvement of *cis*-acting elements regulating the translation of the ATPase mRNAs.

The present study was performed in order to investigate whether cytosolic factors exist that interact selectively with β -mtATPase mRNA. Electrophoretic mobility-shift assays (EMSAs) and UV cross-linking experiments showed that two proteins, here termed β -mtATPase mRNA binding protein (BARB)1 and BARB2, interact with poly(A)⁺ β -mtATPase RNA probes in an interdependent manner. BARB1 is a *bona fide*

poly(A)-binding protein (PABP) that probably achieves mRNA selectivity via interaction with the protein BARB2, which binds to an upstream element in the 3'-untranslated region (UTR) of β -mtATPase mRNA.

EXPERIMENTAL

Animals

Male adult NMRI mice (Eklunds) were kept at room temperature in a 12 h dark/12 h light cycle with food and water *ad libitum*. Animals were maintained for at least 1 week at 4 ± 2 °C before use, in order to ensure good BAT yield.

Preparation of cytosolic cell extract

Cell post-polysomal (S 130) extracts were prepared essentially as described in [7]. Briefly, animals were killed by cervical dislocation and tissues (heart, liver, lung, brain cortex, interscapular BAT and kidney) were dissected and placed in ice-cold buffer A [10 mM Tris/HCl (pH 7.6), 1 mM potassium acetate, 1.5 mM magnesium acetate, 2 mM dithiothreitol, 0.5 mM PMSF and 0.5 $\times$ Complete® (Boehringer)]. The tissues were minced with scissors, rinsed in buffer A, and a 30% (w/v) homogenate in buffer A was prepared (10 strokes in a glass/Teflon homogenizer). The tissue homogenates were subsequently centrifuged at 11000 *g* for 10 min to remove nuclei. The supernatants were layered on top of a 1.5-ml cushion of buffer B [30% (w/v) sucrose in buffer A] and centrifuged at 130000 *g* for 2.5 h at 4 °C (Beckman SW60 rotor). The upper phase was collected and stored at -70 °C. Protein concentrations were determined by the Bradford method [8].

Abbreviations used: mtATPase, mitochondrial ATP synthase; BAT, brown adipose tissue; UTR, untranslated region; EMSA, electrophoretic mobility-shift assay; PABP, poly(A) binding protein; BARB, β -mtATPase mRNA binding protein; 3'- β FBP, β -F₁ ATPase mRNA 3'-UTR binding protein; GPDH, glyceraldehyde-3-phosphate dehydrogenase.

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The nucleotide sequence reported in this paper will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number AF030559.

Table 1 Primers used for preparation of mouse β -mtATPase cDNA

Oligonucleotides used for PCR amplification of Mouse Liver Marathon-Ready™ cDNA (Clontech) in order to prepare mouse β -mtATPase plasmid constructs.

Primer	Sequence
β -288-F	5'-GCA GGG AGA GCA GAC TGG TTT TGG AGG-3'
β -451-R	5'-GAT TCT GCC CAA GGT CTC AGG ACC AAC-3'
β -1582-F	5'-TAA GCT GGC AGA AGA GCA CG-3'
β -1659-F	5'-GAG CTC AGG TTT CCT TCC ATG CAG G-3'
β -1680-R	5'-GCA TGG AAG GAA ACC TGA GC-3'
β -1760-R	5'-GGG GTA TAT ACT TTA TTG AAA ACT T-3'
β -A15-R	5'-TTT TTT TTT TTT TTT GAG GG-3'
AP1*	5'-CCA TCC TAA TAC GAC TCA CTA TAG GGC-3'
GPDH-R*	5'-GAC CAC AGT CCA TGA CAT CAC T-3'

*Control primers supplied with the Marathon-Ready™ cDNA (Clontech).

Preparation of plasmid constructs

Two overlapping fragments of the mouse β -mtATPase cDNA were PCR amplified from Mouse Liver Marathon-Ready™ cDNA (Clontech) using Advantage™ KlenTaq Polymerase mix (Clontech). The first-strand cDNA synthesis was primed with an anchored oligo-dT 30-mer, which means that all cDNAs had a 30-bp poly(A) tail in the 3'-end. The Marathon™ adaptor was ligated to the double-stranded cDNA, on both the 5' and 3' ends. The AP1 primer (Clontech) was complementary to the Marathon™ adaptor and could thus be used both in the amplification of the 3' and 5' end. In order to amplify the 5' end, a reverse primer, β -451-R, was synthesized (see Table 1 for all primer sequences), which was complementary to a mouse brain cDNA (accession number W65207) that was > 95% similar to the rat β -mtATPase cDNA [9]. The resulting 0.5-kb PCR product was subcloned into pBluescript SK⁺ (Stratagene) with T overhangs at the *EcoRV* site, created with *Taq* polymerase as described in [10]. Similarly, use of the forward primer β -288-F and the AP1 primer, now utilized as a reverse primer, yielded a 1.7-kb product, which was also subcloned as above. The two resulting constructs, β -(5'-451) and β -(288-3') respectively, were sequenced using Dye Terminator Cycle sequencing (Perkin-Elmer) in an Applied Biosystems 373 DNA sequencer. β -(288-3') was subsequently used to prepare a number of shorter 3'-end constructs as depicted in Figure 1, which were all subcloned into an appropriately digested pBluescript, except probe A, which was identical with β -(5'-451) and included the Marathon adaptor in the 5'-end. Probe B was a 0.5-kb *PstI/NotI* fragment which included the last 250 bp of the coding region, the entire 3'-UTR and the poly(A) 30-mer directly upstream of a *NotI* site, and was included in the primer used for creating the cDNA library. Probe C was prepared by PCR using (288-3') as template with the forward primer β -1582-F and the reverse primer AP1. The product was blunted with T4 polymerase, digested with *NotI* [in order to terminate the run-off transcript just downstream from the poly(A)], and ligated into a *EcoRV/NotI*-digested pBluescript. Using the same approach, probe D was prepared, using primers β -1659-F and AP1. Probes E and F were *DraI* digests of probes C and D respectively, and were used for *in vitro* transcription without additional subcloning; the *DraI* digest deleted the last 25 bp of the 3'-UTR and the poly(A) tail. Probe G was a *DraI/NotI* fragment of probe B which was subcloned as above. Two additional constructs, J and K, were PCR amplified from probe B with subsequent subcloning into pBluescript, using primers β -1582-F and β -A15-R or β -1582-F and β -1760-R

respectively. A 0.7-kb fragment of the 3'-region of glyceraldehyde-3-phosphate dehydrogenase (GPDH) (accession number M32599) was PCR amplified from the Mouse Liver Marathon-Ready™ cDNA, using primers GPDH-R and AP1, and subcloned as above; this construct (which was used in the experiments shown in Figures 4 and 6) thus included an identical 30-bp poly(A) tail as β -(*PstI-NotI*). This plasmid was used to make two chimaeric probes: in probe H, a 312-bp *ApaI-BlnI* fragment, covering the 3'-end of GPDH but excluding the last 70 bp and the poly(A)-tail, was blunted and inserted in front of probe G. In probe I, position 1582–1682 of probe C was PCR amplified using Pfu polymerase (Stratagene) and primers β -1582-F and β -1680-R (Figure 1B), and the PCR product was subsequently inserted in front of a blunted 100-bp *BlnI-NotI* poly(A)30⁺ fragment of GPDH. A 350-bp *EcoRI* fragment of the 3'-UTR of the full-length mouse P1 isoform of the subunit c of ATPase was isolated from a mouse heart λ gt11 cDNA library (Clontech), sequenced to confirm identity with the known sequence (accession number AA139793) and subcloned as above; this cDNA included an 18-bp poly(A) tail directly upstream of the *EcoRI* site.

In vitro transcription

Template plasmid DNA was linearized with *NotI*, except for subunit c(P1), 3'-GPDH and probe I, which were linearized with *HindIII*, and transcribed with an appropriate phage RNA polymerase [T7 or T3 (Boehringer)] using [α -³²P]CTP (Amersham) with a specific radioactivity of 800 Ci/mmol, diluted 1:1 with unlabelled CTP. The probes were gel purified as described in [10].

RNA binding assay

The binding assay and electrophoresis were performed essentially as described in [11]. Briefly, 10–20 μ g of S130 extract, adjusted to a final concentration of 140 mM KCl, was incubated with 1 μ l of probe [3 fmol; approx. (1–3) $\times$ 10⁴ c.p.m.] and, where indicated, 1 μ l of unlabelled competitor RNA at the indicated concentration added simultaneously. The reactions (total volume of 10 μ l) were incubated for 15 min routinely at 4 °C, but similar results were obtained at room temperature. Afterwards the samples were incubated with 1 μ l (10 units/ μ l) of RNase T1 (Boehringer) for 5 min at room temperature, followed by 1 μ l (5 units/ μ l) of heparin sulphate (Loevens kemiska fabrik) for 5 min at room temperature. The samples were then placed on ice and 2 μ l of loading buffer [50% (v/v) glycerol, 0.5% (w/v) Bromophenol Blue, 0.5% (v/v) xylene cyanol] was added before electrophoresis through a 1-mm 4% (w/v) polyacrylamide non-denaturing gel, run at 7.5 V/cm in 45 mM Tris/borate (pH 8)/1 mM EDTA.

UV cross-linking

After heparin treatment, the samples were UV cross-linked at 254 nm for 15 min on ice, using a Stratalinker (Stratagene) with regular settings. The samples were subsequently boiled for 3 min in 4 M urea/50 mM dithiothreitol/5% (w/v) SDS/0.1% (w/v) Bromophenol Blue (final concentration) and separated by standard SDS/PAGE [10].

Two-dimensional electrophoresis

Following the binding assay and electrophoresis through a 4% (w/v) non-denaturing polyacrylamide gel in the first dimension, the strip of gel containing the mRNA–protein complexes was

Figure 1 Map of mouse β -mtATPase cDNA constructs utilized for riboprobe synthesis

(A) Probes B–G covered the indicated portions of β -mtATPase cDNA. Probes H and I were chimaeric constructs that included the indicated fragments of mouse GPDH cDNA (black rectangles); note that both probe H and I had identical 30-bp poly(A) tails (open rectangle) to the β -mtATPase constructs. Probes J and K were identical with probe C in the 5' end but with a partial or complete deletion of the 30-bp poly(A) tail. (B) Sequence of the 3'-UTR of mouse β -mtATPase mRNA. The underlined sequences indicate the translation stop codon, the *DraI* restriction site, the poly(A) signal and the *NotI* restriction site. The arrow indicates the position of primer β 1680R, which was utilized in preparing probe I.

excised and UV cross-linked at 254 nm for 30 min on ice. The strip was subsequently incubated in 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol for 45 min at room temperature, washed in water and subjected to SDS/PAGE [10] in the second dimension. Dried gels were analysed by phosphorimaging (PhosphorImager; Molecular Dynamics).

RESULTS

Tissue-specific RNA-binding proteins interact with the 3'-UTR of the β -subunit mRNA

In order to investigate whether specific cytosolic factors exist which interact with the β -mtATPase mRNA, cytosolic proteins, isolated from six different tissues, were incubated with labelled RNA probes covering the 5'- or the 3'-UTR of the β -subunit mRNA. Binding was detected using a gel EMSA. In the absence of extract, the riboprobes were degraded by RNase T1, whereas a band shift occurred when the cytosolic extract contained protein(s) capable of binding to and protecting the riboprobe from degradation. With probe A, which covered the short 5'-UTR and the first 0.4 kb of the coding region, no detectable shift was observed with cytosolic extracts from any tissue (results not shown), and no further studies were therefore performed with this probe. In contrast, cytosolic extracts from all tissues tested produced two or three shifted bands with probe D (Figure 2A), a probe that covered the last 120 bp of the 3'-UTR (see Figure 1).

The binding activity was tissue specific (Figure 2A), with the upper, major shift (black arrowhead) being most pronounced in brain and heart, somewhat less prominent in BAT and liver, and weak in kidney and lung. A less prominent lower shift (grey arrowhead) was also present in all tissues. The only tissue extract which exhibited the lower shift more strongly than the upper shift was kidney. Kidney extracts, and to some extent also liver extracts, caused a further minor shift, not detectable in other tissues (open arrowhead).

To characterize the probe D binding proteins, the reaction mixtures were exposed to UV light before resolution on SDS/PAGE. As shown in Figure 2(B), two major RNA–protein cross-linked complexes were formed. These complexes were present in all extracts and had apparent molecular masses of 130 and 75 kDa. These bands were named BARB1 and BARB2. Other minor bands of 45–55 kDa were often visible after UV cross-linking; some of them were expressed in a tissue-dependent manner (kidney), some of them were present in all tissues. These bands were most probably the result of non-specific interaction of cytosolic proteins with the riboprobes (see Figures 3, 4 and 5, where the higher-molecular-mass complexes showed specific displacement with different probes). Small size differences and the existence of double bands were often observed with the cross-linked proteins. These bands were probably due to additional RNase activity in the extracts that reduced the molecular mass of the RNA–protein complex; addition of RNase A, in addition to RNase T1, clearly reduced the apparent molecular

Figure 2 Tissue-specific binding activity of β -mtATPase RNA binding proteins

(A) ^{32} P-labelled riboprobe D was incubated with 20 μ g of S130 extracts from the indicated tissues and, after a 15 min incubation at 4 $^{\circ}$ C, treated with RNase T1 and heparin. The RNA-protein complexes were analysed by EMSA in a 45 mM Tris/borate non-denaturing polyacrylamide gel. The two major bands are indicated with black and grey arrowheads, and the kidney-specific band is indicated with an open arrowhead. (B) As in (A) but UV cross-linking was performed subsequent to heparin treatment and the cross-linked proteins were separated by SDS/PAGE. On the right side, the two major β -mtATPase mRNA binding proteins, BARB1 and BARB2, are indicated. Molecular-mass standards (kDa) are shown on the left. (C) Following the binding assay performed as in (A) and the 4% PAGE in the first dimension (top horizontal lane), the gel strip was cut out and UV cross-linked at 254 nm for 30 min on ice, incubated in 1% SDS and 1% 2-mercaptoethanol for 45 min at room temperature, washed in water and subjected to SDS/PAGE in the second dimension (vertical lanes). The left single lane represents the result of UV cross-linking as in (B). Molecular-mass standards (kDa) are indicated on the left. The positions of BARB1 and BARB2 are also indicated.

mass of BARB1 (results not shown). The occurrence of the radiolabelled bands was dependent upon UV irradiation, and the bands were proteinase K sensitive (results not shown) and could thus be regarded as proteinaceous in nature.

BARB1 displayed similar tissue-specific distribution as did the upper band in the EMSA experiment (Figure 2A) (i.e. binding activity was significantly lower in kidney and lung than in other tissues). The binding activity of BARB2 showed a similar tissue specificity to that of BARB1; only in kidney was there somewhat more BARB2 than BARB1. None of the minor cross-linked proteins could be correlated with the major EMSA shift.

In order to verify that the two BARB proteins were responsible for the major shift in the EMSA, we performed a two-dimensional analysis. Here the EMSA was carried out before cross-linking, after which separation on an SDS/polyacrylamide gel was performed (Figure 2C). It is very clear that the upper, major EMSA shift could be resolved into bands apparently identical with the BARB1 and BARB2 proteins; no other proteins were evident. This indicates the existence of a native BARB1-BARB2-mRNA complex. In contrast, the lower, weaker EMSA shift contained only BARB2, indicating that BARB2 also could

Figure 3 Mapping of binding elements for BARB1 and BARB2

(A) S130 extracts (10 μ g), isolated from mouse brain, were incubated for 15 min with the indicated ^{32} P-labelled probes, treated with RNase T1 and heparin sulphate, UV cross-linked and analysed by SDS/PAGE. The positions of BARB1 and BARB2 are indicated on the left and molecular-mass standards (kDa) are shown on the right. (B) As for (A) except that samples were analysed by regular EMSA. The two major bands are indicated by arrowheads.

bind to the mRNA without BARB1 (but no evidence for the reverse was found). It was thus feasible to use the cross-linked complexes to further characterize the native complexes.

BARB1 and BARB2 recognize different regions in the β -subunit mRNA 3'-UTR

In order to map the binding region for the RNA-binding proteins that interact with the β -subunit mRNA, we compared the cross-linking pattern of a series of different probes spanning the coding region and the 3'-UTR (outlined in Figure 1A), using brain cytosolic extracts. Whereas probes B, C and D (Figure 3A, lanes 1–3) were able to cross-link with both BARB1 and BARB2, the two shorter probes, E and F, cross-linked only with BARB2 (Figure 3A, lanes 4 and 5), indicating that the region downstream of position 1734 (see Figure 1B), i.e. the poly(A) signal and the 30-bp poly(A) tail, was required for BARB1 binding. Surprisingly, probe G, which covered only that region, failed to interact with anything (Figure 3A, lane 7), thus suggesting that, although this region was essential, it was not sufficient for specific BARB1 interaction. In addition, probe H, where the 3'-end of mouse GPDH had been ligated upstream of probe G, was still unable to interact with BARB1 and BARB2; the weak bands that did cross link with probe H could not be competed out with a 250-fold molar excess of unlabelled probe D (results not shown). Two other probes with deletions in the 3'-end were also tested for binding. Probe J, which was identical with probe C but with only a 15-bp poly(A) tail, interacted with both BARB1 and BARB2, whereas probe K, which completely lacked the poly(A) 3' end but otherwise corresponded to probe C, failed to interact with BARB1. It can be observed (Figure 3A, lane 7) that the BARB1 complex with probe J was approx. 10 kDa smaller than the BARB1 complex with probe C. This difference is to be

Figure 4 Characterization of the specificity of BARB1 and BARB2 binding

(A) S130 extracts (10 μ g), isolated from mouse brain, were incubated with ^{32} P-labelled probe D together with the indicated unlabelled competitor RNA in 5-fold, 50-fold or 250-fold molar excess, shown by an open wedge above the three lanes corresponding to each competitor. In lane 1, 14 and 18, no competitor RNA was used. Binding reactions were analysed after UV cross-linking, as described in Figure 3(A). The positions of BARB1 and BARB2 are indicated on the right and molecular-mass standards (kDa) are shown on the left. (B) As for (A) but, in lane 2, no competitor RNA was used. Binding reactions were analysed by EMSA. The two major bands are indicated by arrowheads.

expected in view of the difference of the 15-bp poly(A) between the two probes. In summary, these findings suggest that: (1) BARB1 binding involves interaction with the poly(A) tail of the β -subunit mRNA, but also requires binding of BARB2, (2) BARB2 can bind without BARB1 and (3) a 75-bp element covering nucleotides 1659–1734 is sufficient for BARB2 binding.

In order to further confirm (see Figure 2C) that the results with the BARB1 and BARB2 proteins shown in Figure 3(A) correlated with relevant band shifts in the EMSA, the various probes were also run under non-dissociating, non-cross-linking conditions. As shown in Figure 3(B), only the probes that interacted with both BARB1 and BARB2 produced the two major shifts, even though probes E and F clearly interacted with BARB2. This would suggest that, in the absence of BARB1, BARB2 interacted only weakly with the β -mtATPase mRNA, enough to allow cross-linking but not with sufficiently high affinity to allow probe retardation in the EMSA. The results showed also that simultaneous binding of both BARB proteins to the mRNA required not only the poly(A) tail, but also upstream sequences present in probe F, i.e. probe D was the minimum sequence sufficient to bind both the BARB proteins.

To provide an insight into the binding specificity of the two BARB proteins, we tested competition of probe D binding with increasing amounts of unlabelled probes, corresponding to different regions of the β -subunit 3'-UTR, with non-specific RNAs [3'-UTR with poly(A) extensions], or with different homoribopolymers.

Cross-linking of BARB1 and BARB2 to probe D was reduced by a 5-fold molar excess of unlabelled probe B, C or D (Figure 4A, lanes 1–7 and 18–21; Figure 4B, lanes 2–5). However, even a 250-fold excess of unlabelled probe F was not able to effectively compete for either BARB1 or BARB2 binding (Figure 4A, lanes

Figure 5 Characterization of the poly(A) specificity of BARB1 binding

(A) S130 extracts (10 μ g), isolated from mouse brain, were incubated with ^{32} P-labelled probe D and, as competitor RNA, 1 ng, 10 ng or 100 ng of unlabelled ribopolymers was used (shown by open wedges above the lanes). Binding reactions were analysed by UV cross-linking as described in Figure 3(A). The positions of BARB1 and BARB2 are indicated on the right, and molecular-mass standards (kDa) are shown on the left. In lanes 15–18, ^{32} P-labelled probe F was used instead of probe D. In lanes 1, 11 and 15, no competitor RNA was used. (B) As for (A) but binding reactions were analysed by EMSA. Two major bands are indicated by arrowheads. In lane 2, no competitor RNA was used.

8–10; Figure 4B, lanes 6–8), despite the ability of this probe to interact with BARB2 in cross-linking studies (see Figure 3A), thus strengthening the previous suggestion that BARB2 interacts with probe F with low affinity in the absence of BARB1. The 3'-UTRs of the subunit c of ATPase or of GPDH, which included adenylate 18-mers or 30-mers respectively in their 3'-ends, were also not able to effectively compete with probe D for binding of BARB1 and BARB2 (Figure 4A, lanes 11–17; Figure 4B, lanes 9–14). At the highest concentration of the unlabelled GPDH probe (250-fold excess; Figure 4A, lane 17 and Figure 4B, lane 14), competition with BARB1 binding was evident. However, since BARB1 most probably has affinity for the poly(A) tail, some titration of BARB1 may be expected merely by addition of high amounts of poly(A)⁺ mRNAs.

The inability of the poly(A)-deficient probes (E, F and K) to interact with BARB1 in the cross-linking studies strongly supported the suggestion that BARB1 was indeed a β -mtATPase-mRNA-selective poly(A)-binding protein. Competition binding reactions with probe D, using unlabelled homoribopolymers of heterogeneous length, are shown in Figures 5(A) and 5(B). It is clear that BARB1 binding was indeed titrated out by addition of an excess of both poly(A) and poly(U), by analogy with the two poly(A)-binding proteins PABP and iPABP (inducible 80-kDa PABP) [12] (see the Discussion section); high affinity for poly(U) thus appears to be a common feature amongst PABPs. BARB2, on the other hand, was markedly less affected by poly(A). However, in the EMSA, both major bands were completely displaced by the addition of increasing amounts of poly(A), and there were no residual bands that could be attributed to BARB2 binding alone (Figure 5B, lanes 2–5). These results are in agreement with those from the EMSA and cross-linking (see Figures 3A and 3B), where the removal of the poly(A) tail completely abolished binding of both BARB1 and BARB2 in the

Figure 6 Specificity to BARB1 and BARB2 conferred by a 22-bp upstream element

(A) S130 extracts (10 µg) isolated from mouse brain were incubated either with ³²P-labelled 3'-end GPDH riboprobe (lanes 1 and 2), probe B (lanes 3–5) or probe I (lanes 6–10). As unlabelled competitor (comp.) RNA, 100 ng of either poly(A) (lanes 1 and 7), poly(U) (lane 8), probe C (lanes 4 and 9) or probe D (lanes 5 and 10), were used. Binding reactions were analysed by UV cross-linking as described in Figure 3(A). The positions of BARB1 and BARB2 are indicated on the right and molecular-mass standards (kDa) are shown on the left. (B) S130 extracts (10 µg), isolated from mouse brain, were incubated with ³²P-labelled probe D that was either untreated (lane 1) or denatured at 95 °C (lane 2) or 70 °C (lane 3) for 5 min before addition to the binding reaction. Binding reactions were analysed by UV cross-linking as described in Figure 3(A). The positions of BARB1 and BARB2 are indicated on the right. (C) Comparison of the 3'-UTR in β-mtATPase cDNAs of mouse, cow and human β-mtATPase mRNA using the PileUp program in the GCG package. The 22-bp element and the poly(A) signal are boxed and the uridylyte core is indicated by boldface letters.

EMSA. Poly(G) or poly(C) were both completely ineffective in competing with probe D binding, but, unexpectedly, poly(U) competed with some efficiency for BARB2 binding. To determine whether the competition by poly(U) for the binding of BARB2 was merely related to steric hindrance due to possible annealing of the unlabelled homoribopolymer with the poly(A) tail, we utilized the ability of probe F to interact with BARB2 in cross-linking experiments. As shown in Figure 5(A) (lanes 15–18), poly(U) was also able to compete with probe F for BARB2 binding, indicating that the BARB2 binding element is composed of U-rich sequences. Furthermore, annealing between competitor

RNA and probe is unlikely, because all the binding experiments were performed at 4 °C.

A 22-bp element with a uridylyte core confers specificity to BARB2

From the foregoing experiments, we concluded that BARB1 is a *bona fide* PABP with specificity for β-mtATPase mRNA, and that BARB2 most probably interacts with a U-rich region in probe F. However, the 75-bp probe F, which is the shortest probe that interacts with BARB2 (Figure 1B), lacks any very long stretches of uridylyte residues akin to the AU-rich elements of unstable transcripts [13]. The longest region of uridylyte residues within the 3'-UTR of the β-mtATPase mRNA is the sequence UUUCCUU found at positions 1670–1676. This sequence lies within probe F and could thus confer the poly(U) responsiveness on BARB2 binding. To address the putative importance of this element, we made a fusion probe, where β-subunit-specific sequences from positions 1582–1680, which included the UUUCCUU core, were inserted in front of the last 80 bp of GPDH, including its 30-bp poly(A)-tail. The cross-linking pattern of this probe (probe I) was then compared with the cross-linking pattern of a 0.7-kb 3'-GPDH probe alone. As shown in Figure 6(A), none of the multiple proteins that cross-linked with the GPDH probe showed any poly(A) sensitivity (Figure 6A, lanes 1 and 2), nor could their binding be competed with probe B, C or D (results not shown). However, the fusion probe (probe I) cross-linked with two proteins of the same sizes as BARB1 and BARB2, and with an additional non-specific protein. It was possible to compete out the binding of BARB1 and BARB2 proteins by the addition of poly(A), poly(U), probe C or D (Figure 6A, lanes 6–10). Thus it seems likely that the specificity for both BARB1 and BARB2 lies within region 1582–1680; furthermore, since probe D, which appeared to be sufficient for the stable binding of BARB1 and BARB2, starts at position 1659, this strongly implies that the 22 bp from nucleotides 1659–1680 (also present in probe I) are sufficient to confer specificity to the poly(A) tail, and thus to facilitate BARB1 binding. Again, the ability of poly(U) to compete for BARB2 binding (Figure 5A) supports the importance of the uridylyte core within this region for the RNA–protein interaction. Additional support may be found in a comparison of the 3'-UTRs of β-mtATPase mRNA from three different species (Figure 6C), in that some evolutionary conservation of the uridylyte core between mouse (the present study), cow [14] and man [15] can be noted. To examine the possibility that recognition of the poly(A) binding site by the BARB1 protein, where the poly(A) tail annealed to the UUUCCUU element, involved secondary structures, we denatured probe D at 95 °C or 70 °C for 5 min before binding. As shown in Figure 6(B), probe denaturation had no effect. This indicates that BARB2 may confer specificity to BARB1 by protein–protein interaction rather than by secondary structural motifs in the RNA.

DISCUSSION

In the present study, evidence for the existence of two proteins, designated BARB1 and BARB2, which interact with the 3'-UTR of β-mtATPase mRNA is given. BARB1 is a novel PABP that has selective affinity for the poly(A) tail of β-mtATPase mRNA; other poly(A)⁺ mRNAs failed to compete with BARB1. The selective nature of BARB1 binding is achieved not through the poly(A) tail but by the interaction of BARB1 with BARB2, which binds to a specific element with a uridylyte core located 50 bp downstream of the stop codon. Insertion of this element into the 3'-UTR of the GPDH results in BARB1 interaction with

the poly(A) tail of the chimaeric riboprobe. Possible interactions of BARB1 and BARB2 could be discerned using the MFOLD program, which showed the 3'-UTR of β -mtATPase mRNA in its most stable conformation. Interestingly, all the uridylate residues that we believe interact with BARB2 are located in a single-stranded region; single-stranded RNA is more likely to form sequence-specific RNA-protein interactions [16].

BARB1 and other PABPs

Several PABPs have been described to date. The classical 70-kDa PABP has been isolated from a number of sources and shows extensive evolutionary conservation [17]. The more recently discovered inducible 80-kDa PABP, iPABP, was cloned and isolated in a screen for genes activated by phorbol esters in human T lymphocytes [12]. In addition, a 49-kDa nuclear PABP (PAB II) has been isolated from calf thymus, and this was shown to facilitate efficient polyadenylation of growing poly(A) tails [18]. BARB1 is probably functionally a fourth member in this class of PABPs. However, the above-mentioned PABPs are believed to bind any poly(A)⁺ mRNA. In contrast, BARB1 appears to selectively interact with β -mtATPase mRNA, compared with other mRNAs tested in the present study (although we cannot exclude the possibility that BARB1 is also able to interact with certain other species of mRNA, which we have not identified).

BARB2 and other β -mtATPase mRNA binding proteins

Both BARB1 and BARB2 eluded detection in a previous investigation of β -mtATPase mRNA-binding factors [19]. One reason could be that a 15-bp poly(A) tail is a minimum length which will allow binding of the BARB1 protein and that the RNA probes used in the earlier study [19] did not have a sufficiently long poly(A) extension in the 3'-end (only 13 bp). The protein described therein [19], β -F₁ ATPase mRNA 3'-UTR binding protein (3'- β FBP), is perhaps identical with the kidney-specific 45-kDa protein shown in Figure 2(B). This is supported by the fact that the highest level of 3'- β FBP was observed in kidney [19], and that the estimated molecular masses of the kidney-specific RNA-protein complex described in the present work and 3'- β FBP were similar (52 kDa versus 45 kDa).

Conclusions

BARB1 is, to our knowledge, the first poly(A)-binding protein described that demonstrates a preference for a specific mRNA. Interestingly, this novel poly(A)-binding protein achieves its specificity for β -mtATPase mRNA by interacting with another protein, BARB2, which binds to an upstream element of the mRNA. These findings may not only be of importance in the

study of mtATPase and mitochondrial biogenesis, but may also engender a novel theory for the role of *cis*-acting elements in mRNA. Bernstein et al. [20], suggested that *cis*-acting elements of unstable transcripts interfered with PABP binding to the poly(A) tail, resulting in de-adenylation and subsequent mRNA decay. In an extension of this theory, the present findings suggest that proteins binding to *cis*-acting elements in the 3'-UTR may attract selective poly(A)-binding proteins, e.g. BARB1, that appear to displace PABP binding. It is very likely that a number of mRNA-specific PABPs may have eluded discovery because such earlier studies were often performed with RNA probes without a sufficiently long poly(A) 3'-tail. Further studies, aimed at isolating and characterizing the BARB proteins, will be important in order to investigate which of these proteins have a role in the post-transcriptional regulation of ATP synthase biosynthesis [21–24].

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