The mRNA-binding protein COLBP is glutamate dehydrogenase

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Abstract Expression of the liver-type isopeptides of cytochrome *c* oxidase is regulated post-transcriptionally. An RNA-binding activity has been found in only those cells where the liver-type subunits are detected. This binding protein, termed COLBP, recognises sequences or structures within the 3′-untranslated regions of transcripts encoding these liver-type isopeptides and has been implicated in the modulation of mRNA expression. We now show by subcellular fractionation, immunocompetition, UV-crosslinking and shift-Western studies that the metabolic enzyme glutamate dehydrogenase, previously reported as being able to bind RNA, is the cytochrome *c* oxidase transcript-binding protein, COLBP.

Key words: Glutamate dehydrogenase; RNA-binding protein; Cytochrome *c* oxidase; Post-transcriptional regulation; Isoform regulation

1. Introduction

The expression of many mRNA species is regulated by proteins binding to sequences or structures within those transcripts. These binding sites, or response elements, are often located in mRNA untranslated regions and interaction with *trans*-acting binding factors may impart rapid degradation, stabilization or translational silencing of the bound mRNA [1–3]. We are currently investigating the mechanisms underlying the post-transcriptional regulation of the liver-type isoforms of cytochrome *c* oxidase (COX). Several subunits of mammalian COX are found as either one of two tissue-specific isoforms referred to as either the heart (H-) or liver (L-) type [4,5]. Numerous reports have shown that whereas the H-isotypes are regulated at the level of transcription, the L-type transcripts are present at varying levels in all tissues, irrespective of the isopeptide's presence in the enzyme complex [6–10]. Using radiolabelled COX L-type transcripts as substrates, we recently demonstrated, both by gel mobility shift and UV-crosslinking analyses, the formation of an RNA–protein complex in cell lysates from only those cell types where the L-type isopeptides are detected [11]. This binding protein has therefore been implicated in regulating mRNA expression possibly by increasing mRNA stability. In support of this hypothesis, we also observed that mitochondrial stress causes a concomitant increase in COX L-type transcript stability and binding protein activity [12]. Attempts to isolate the binding protein (termed COLBP or cytochrome *c* oxidase L-form transcript-binding protein) were initially complicated by the appearance, after ion exchange chromatography, of a second binding complex with a lower mobility on nondenaturing gels. Purification and protein sequence data assigned this second activity to glutamate dehydrogenase [13]. Competition assays showed the enzyme did not bind homoribopolymers, single stranded DNA or tRNA.

Using a combination of subcellular fractionation, immunocompetition, UV-crosslinking and shift-Western studies we now show that there is only one identifiable COX L-type transcript binding protein, the mitochondrial matrix enzyme glutamate dehydrogenase.

2. Materials and methods

2.1. Preparation and subfractionation of mitochondria and measurement of enzyme activities

Mitochondria were prepared from fresh liver tissue by homogenization and differential centrifugation using STE (250 mM sucrose, 2 mM EDTA, 10 mM Tris(Cl), pH 7.4, 0.5 mM DTT/PMSF) as medium. For the final wash, mitochondria were resuspended in STE binding buffer (STEBB; as STE but 0.2 mM EDTA, 40 mM NaCl, 2 mM $MgCl_2$). Samples of homogenate and postmitochondrial supernatant were dialysed against STEBB or standard binding buffer (SBB; 20 mM Tris(Cl), pH 7.6, 0.2 mM EDTA, 40 mM NaCl, 2 mM $MgCl_2$, 10% glycerol (v/v), 0.5 mM DTT/PMSF). Equal volumes of 1.5% (w/v) digitonin and mitochondrial suspension at various protein concentrations in STEBB were mixed on ice followed by 3-fold dilution after 15 min. Mitoplasts were pelleted, and suspended with STEBB, 1% Triton X-100 (v/v) in a volume proportional to the starting amount of mitochondrial protein. Supernatants were concentrated accordingly before adding Triton X-100 to 1% (v/v). Mitochondrial proteins were separated into soluble and integral membrane proteins by means of the temperature-induced phase separation in Triton X-114 [14]. Enzymatic activities were measured spectrophotometrically essentially as described [15–17]. For modified GMS-assay, the prepared mitochondria in STEBB were checked for integrity by oxygen electrode measurement [18] and respiratory control ratios were routinely between 3–5.

2.2. Preparation of ^{32}P-labelled COX L-type transcripts, S-100 cytosolic extracts, and gel mobility shift analyses

High specific activity ^{32}P-labelled RNA was transcribed from linearised plasmids pCOL8-356, pBSF3-2 or pCOX7a.22 as previously described [11,13,19] using an in vitro transcription kit (Stratagene). In this way, 5′-truncated bovine COX subunit VIII-L (BCOL8), and VIIa-L (BCOL7a), or full-length human COX subunit VIIa-L (HCOL7a) transcripts were produced. Preparation of bovine liver S-100 cytosolic extracts and standard GMS-analysis were essentially as previously described [13]. Protein preparations were incubated with ^{32}P-labelled COX L-type transcripts in the presence of poly(A) and -(C) (2.5 μg each), and 1% (v/v) Triton X-100 (Fig. 1 only). Binding mixtures (10 μl) were based on STEBB or SBB, and were resolved by non-denaturing 4% PAGE. Quantitation of binding complex intensity was by PhosphorImager analysis using ImageQuant software (Molecular Dynamics).

Binding of RNA to intact mitochondria was analysed as follows.

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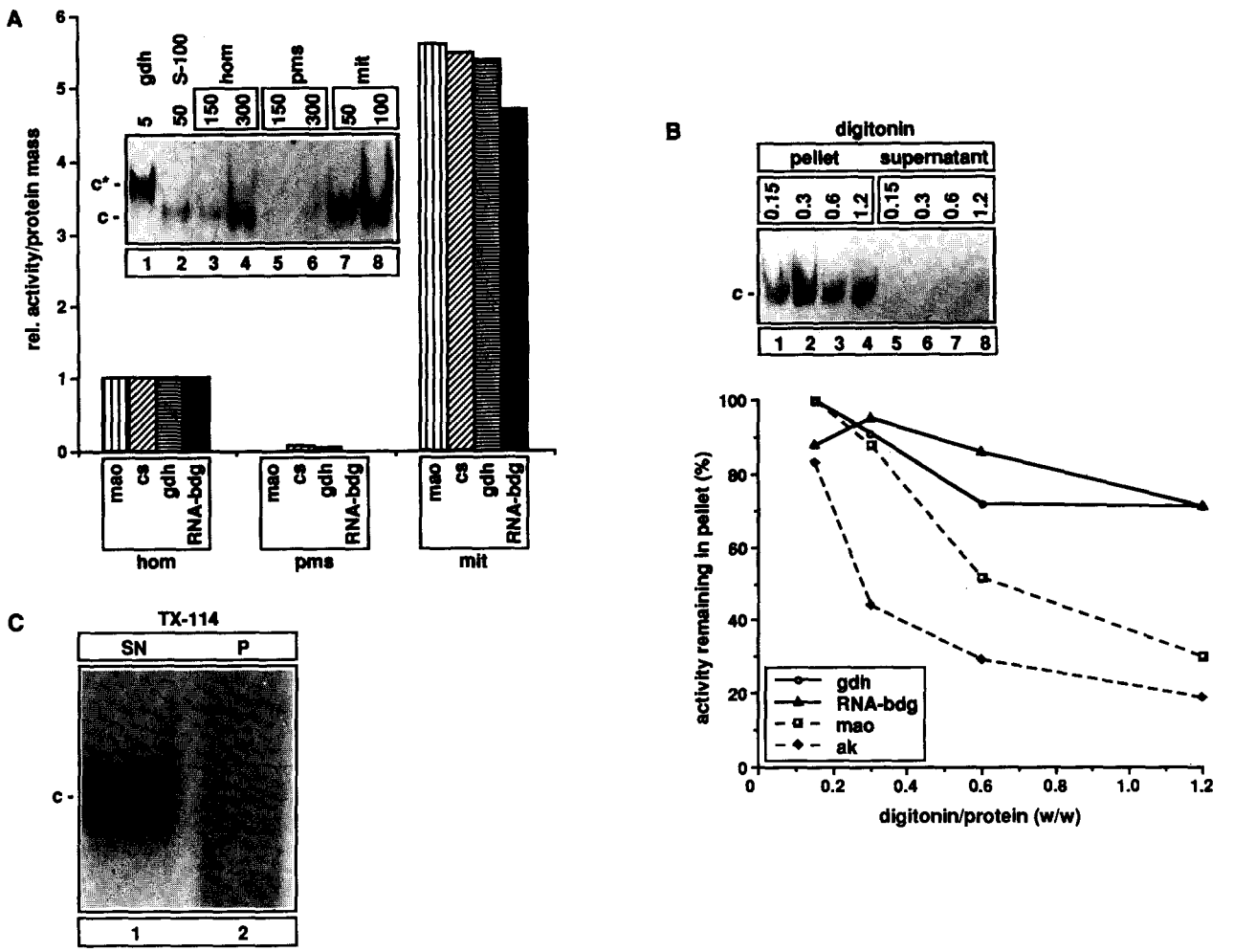

Fig. 1. COX L-type transcript binding protein behaves as a soluble mitochondrial matrix protein. (A) Homogenate (hom), postmitochondrial supernatant (pms), and mitochondrial fraction (mit) from bovine liver tissue were analysed for monoamine oxidase (mao), citrate synthase (cs), and glutamate dehydrogenase (gdh) activities, as well as RNA-binding (RNA-bdg). GMS-assays of these fractions using ^{32}P-labelled BCOL7a as substrate are shown (inset, lanes 3–8), alongside purified gdh (lane 1) and S-100 extract (lane 2) for comparison. Protein amounts used are stated above the lanes in μg. The bar chart gives the activities per protein mass relative to the homogenate. (B) Digitonin-treated mitoplast pellets (lanes 1–4) and corresponding supernatants (lanes 5–8), equivalent to 190 μg mitochondrial protein, were analysed by GMS-assay with ^{32}P-labelled BCOL7a as substrate. The digitonin to protein ratio used is indicated above the lanes. Fractions were also assayed for marker enzyme activities (adenylate kinase; ak) and RNA-binding. The fraction of activity remaining in the mitochondrial pellet is displayed as % of total. (C) Aliquots, corresponding to 60 μg protein, of aqueous supernatant (SN; lane 1) and detergent-rich pellet (P; lane 2) derived from Triton X-114 treatment of bovine mitochondria were analysed by GMS-assay using ^{32}P-labelled HCOL7a as substrate.

Mitochondrial preparations in STEBB were preincubated for 5 min on ice with poly(A) and -(C) (8 or 25 μg each), and binding reactions with ^{32}P-labelled RNA (500,000 cpm) were carried out at room temperature for 15 min. Consecutive treatment with RNase T1 (40 or 100 units) and heparin (100 or 250 μg) was for 5 min each at room temperature. Triton X-100 (0.45% v/v) was added to the reactions at the indicated stages during the incubation and the solutions (final volume 55 μl) gently mixed until homogeneous. Analysis by non-denaturing PAGE was as given above. Mitochondrial integrity during the procedure was monitored by centrifuging control incubations and assaying enzyme activities in mitochondrial pellet and supernatant.

2.3. Production of anti-gdh antisera, affinity purified antibodies, shift-Western blotting and immunoprecipitation

Rabbits were inoculated subcutaneously with 8.9 nmol bovine glutamate dehydrogenase (Sigma Type VI) in 500 μl of 1:1 phosphate buffered saline/Freund's complete adjuvant. Three equimolar boost inocula with incomplete adjuvant were performed over a two-month period before exsanguination after four months. Affinity purification of anti-gdh antibodies was achieved using glutamate dehydrogenase immobilized on CNBr-sepharose 4-B (Sigma) following standard techniques. For shift-Western analysis, an adaptation of procedures published for DNA-binding proteins [20,21] was employed. Standard GMS-assays were performed with S-100 extract. The wet gel was exposed to a PhosphorImager screen before transfer to Immobilon-P membrane (Pharmacia) and hybridization with polyclonal anti-gdh antisera. Radiolabelled RNA was not retained on the membrane after transfer. Detection of bound antibody was by enhanced chemiluminescence (ECL-kit, Amersham).

For immunoprecipitation, GMS assays were performed with S-100 extract in SBB buffered with triethanolamine (50 mM; pH 7.6) after a preclearing step with protein-A agarose (Sigma). The reactions were UV-irradiated for 20 min at room temperature (UV-Stratalinker 2400, Stratagene), followed by digestion with 6 μg RNase A for 10 min at room temperature. Samples were then diluted to 50 μl with PBS, added to antibodies prebound to protein-A agarose (3 μl bead volume), and incubated, rotating, for 2 h at 4°C. After removal of the supernatant, the agarose beads were washed and resuspended in PBS. Supernatants

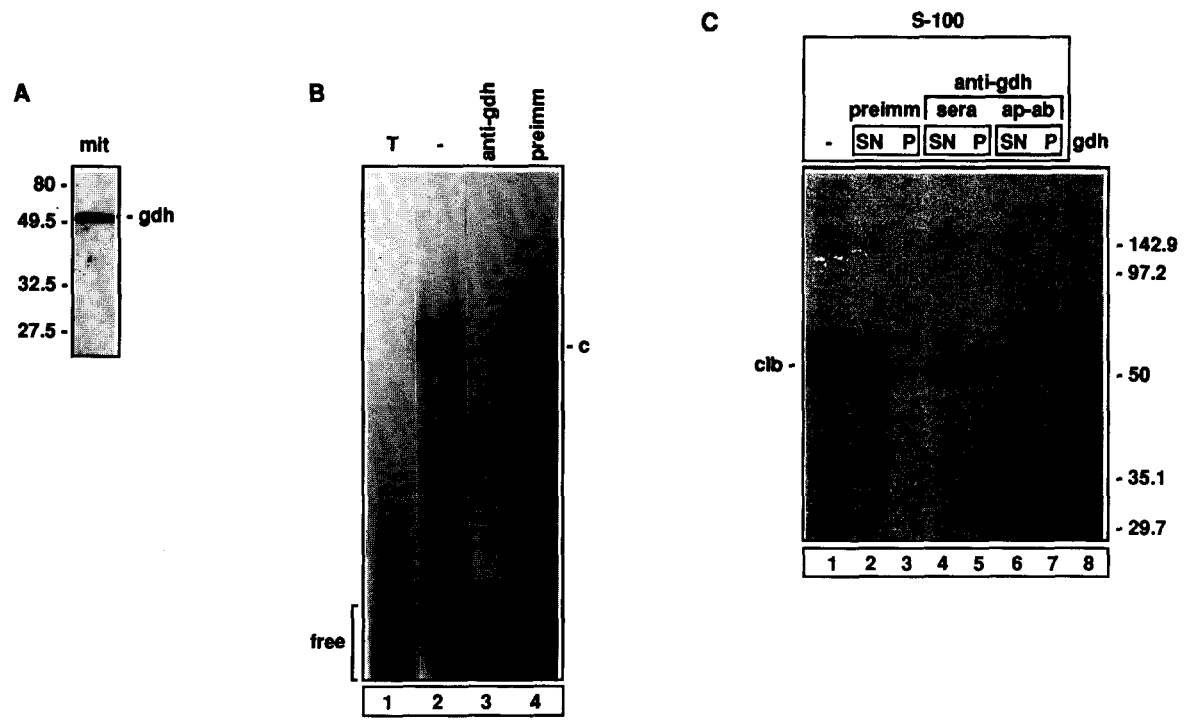

Fig. 2. Anti-gdh antibodies recognize COLBP and compete for complex formation. (A) Bovine liver mitochondrial protein (25 μg) was resolved by 10% SDS-PAGE and blotted to Immobilon-P membrane. A single immunoreactive band was detected with anti-gdh antisera (1:200 v/v). Positions of prestained molecular weight markers are indicated. (B) S-100 extract protein (50 μg) was preincubated with 115 μg polyclonal anti-gdh antisera (lane 3), or 140 μg preimmune sera (lane 4) for 10 min at 25°C, followed by GMS-assay using ^{32}P-labelled HCOL7a as substrate. A control without serum addition (lane 2), and untreated RNA (lane 1) are also shown. (C) Purified glutamate dehydrogenase (5 μg, lane 8) or S-100 extract (200 μg) were incubated with BCOL7a as per GMS-analysis and subjected to UV-crosslinking and RNase A digestion. Samples were immunoprecipitated with: 6.5 μg affinity purified anti-gdh antibody (apa; lanes 6, 7), 320 μg anti-gdh antisera (lanes 4, 5) or preimmune serum (lanes 2, 3). Pellets (P) and supernatants (SN) were subjected to 10% SDS-PAGE. A control incubation without immunoprecipitation (5 μg gdh, lane 8) is also shown. (D) S-100 extract protein (50 μg; lanes 2, 3 or 100 μg; lanes 4–8) were preincubated with 1 mM of the indicated nucleotide(s) for 10 min on ice followed by GMS-assay using BCOL7a as substrate. Control incubations without ^{32}P-labelled RNA (lanes 2, 3) and/or nucleotide addition (lanes 2, 4) are also shown, as is the mobility of untreated RNA (lane 1). The left panel (RNA) shows the radioactive image of the gel. The right panel (protein) represents the immunoblot of the same gel using anti-gdh antisera.

and the bead-bound proteins were subjected to 10% SDS-PAGE, followed by PhosphorImager analysis.

3. Results and discussion

We previously demonstrated that purified bovine glutamate dehydrogenase could form a complex with COX L-type transcripts [13]. A different electrophoretic mobility was noted for the RNA–protein complex observed with S-100 extract, suggesting the extract contained a distinct RNA-binding protein, the as yet unidentified COLBP. In an attempt to study COLBP in the absence of glutamate dehydrogenase, the mitochondrial enzyme was removed by subcellular fractionation of bovine liver tissue. Surprisingly, as illustrated in Fig. 1A, COLBP activity was found only in the mitochondrial fraction. To detect the submitochondrial location of this activity, the outer mitochondrial membrane was selectively solubilized with digitonin. Matrix (gdh), intracristal space (ak) and outer membrane (mao) markers were assayed as detailed in the legend to Fig. 1B. Even after release of 81% adenylate kinase and 70% monoamine oxidase, COLBP activity remained with the mitoplasts. The solubility of proteins in Triton X-114 is routinely used to confirm their integral membrane nature. The detergent liberates all binding activity into the aqueous phase (Fig. 1C). Thus, all the cytoplasmic RNA-binding activity is limited to the mitochondrial fraction and the binding protein behaves like a soluble mitochondrial matrix protein.

Glutamate dehydrogenase and COLBP clearly colocalize, but on non-denaturing gels the RNA–protein complex formed with purified glutamate dehydrogenase exhibits a decreased mobility (Fig. 1A). Furthermore, only the faster migrating binding-complex is resolved using mitochondrial extracts. Assuming glutamate dehydrogenase was synonymous with or integral to COLBP, the discrepancy in mobility could be due to the following: a difference in conformation, differential protection of the bound RNA molecule against RNase T1, an increased charge density due to post-translational modification or interactions with small, acidic molecules such as cardiolipin which may be lost during purification of the enzyme. To test whether COLBP was, or contained glutamate dehydrogenase, polyclonal antibodies were prepared against bovine glutamate dehydrogenase (Fig. 2A). Formation of any detectable RNA–protein complex was prevented by preincubation of S-100 extract (Fig. 2B, lane 3) or purified enzyme with anti-gdh antisera (data not shown), but was unaffected by similar amounts of preimmune serum (lane 4). We have previously demonstrated

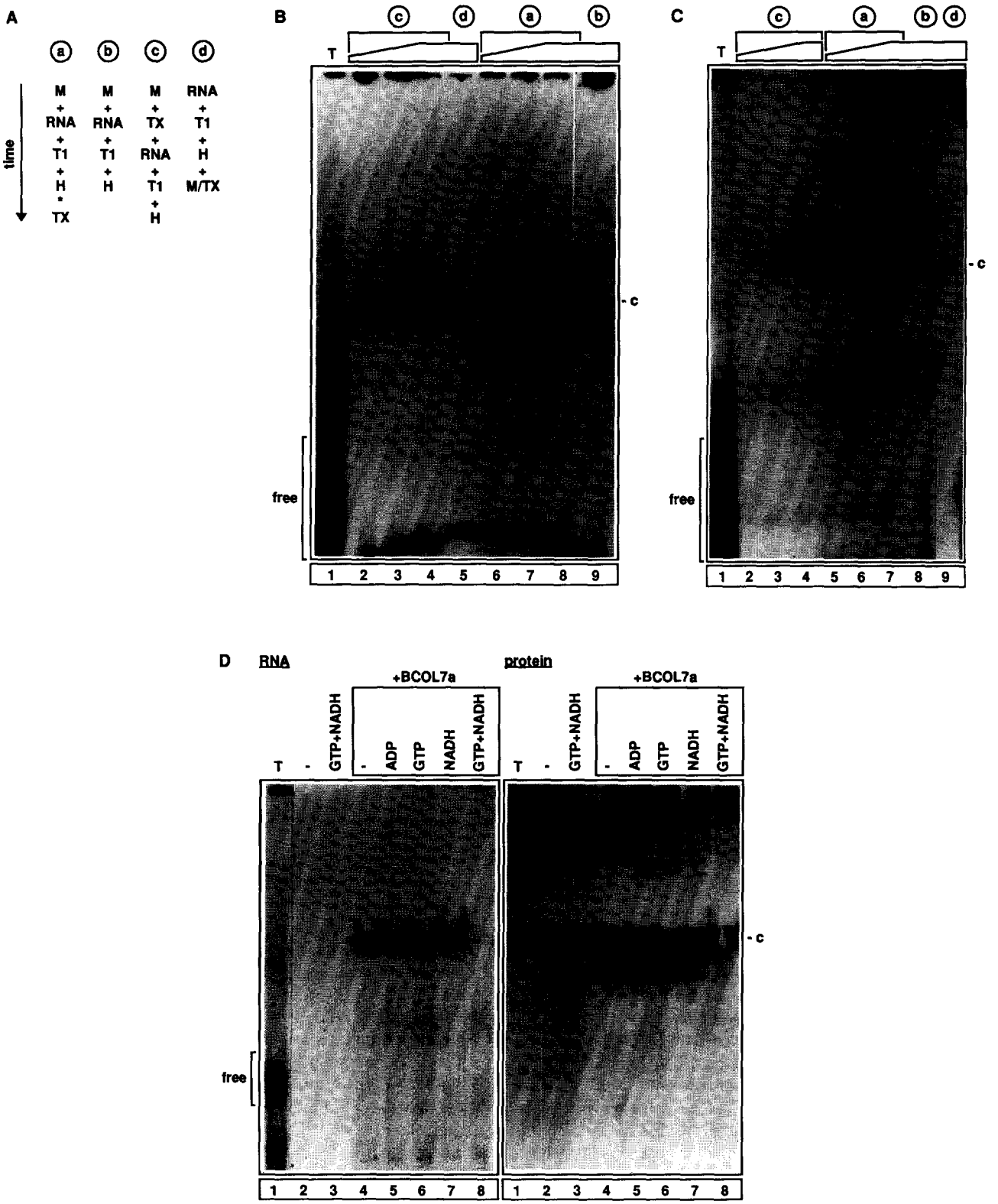

Fig. 3. Glutamate dehydrogenase in intact mitochondria can interact with exogenously added COX transcripts. Formation of RNA–glutamate dehydrogenase complexes was assayed with intact mitochondria prepared from either bovine or rat liver. (A) Schematic representation indicating order (a, b, c or d) of component addition (M = mitochondria; RNA = ^{32}P-labelled COX L-type RNA; T1 = RNase T1; H = heparin; TX = Triton X-100). (B) Bovine liver mitochondria (100 μg, lanes 2, 6; 300 μg, lanes 3, 7; or 500 μg protein, lanes 4, 5, 8, 9) were incubated with ^{32}P-labelled BCOL8 as substrate. The order of addition of all reaction components is indicated above the lanes (cf. A). Recovery of mitochondria from a control incubation (scheme b without ^{32}P-labelled RNA) showed loss of 3.8% gdh-, and 2.1% ak activity. No loss of mao was measurable. (C) GMSanalysis with rat liver mitochondrial preparations (100 μg, lanes 2, 5; 300 μg, lanes 3, 6; 500 μg 4, 7–9) as in B, but using ^{32}P-labelled BCOL7a as substrate. The control incubation according to scheme b showed loss of 1.2% gdh- and 3.7% ak activity.

that COLBP can be visualized by SDS-PAGE after UV-crosslinking to ^{32}P-labelled COX mRNAs and RNase digestion [11]. After similar treatment of S-100 extract or purified glutamate dehydrogenase, a protein of identical size is observed (Fig. 2C, lanes 1,8). This protein can be immunoprecipitated from liver homogenate by affinity-purified anti-gdh antibodies (lanes 6,7).

Further evidence for glutamate dehydrogenase and COLBP being identical proteins was provided by shift-Western analysis. Bovine liver extract and radiolabelled BCOL7a transcript were mixed and subjected to GMS-analysis as detailed in the legend to Fig. 2D. Complex formation was initially identified by PhosphorImager analysis of the wet gel (Fig. 2D, left panel), after which the protein was transferred to a membrane and assayed by standard Western blotting with anti-gdh antisera (right panel). The immunoreactive and radiolabelled band clearly co-migrates (cf. lane 4, left and right panels). Glutamate dehydrogenase activity is known to be regulated by several nucleotide effectors [22], levels of which fluctuate dependent on the energy state of the mitochondria. Shift Western analysis of liver S-100 extracts assayed for RNA-binding in the presence of several effectors shows that binding is inhibited by a combination of 1 mM GTP and NADH but by neither independently (lanes 6–8). A partial inhibition of complex formation in the presence of 1mM ADP was also consistently noted (lane 5). As the formation of large concentration-dependent multipolymer species of glutamate dehydrogenase can be induced by varying effector levels [23], it was a formal possibility that the absence of complex formation with the combination of GTP and NADH may have been caused by generating these multipolymer species. Western analysis, however, revealed that the absence of complex formation in 1mM GTP/NADH was not due to decreasing levels of the binding species (right panel, lane 8).

Taking the data presented in Figs. 1 and 2, we conclude that only one protein can be shown to interact with the 3′-untranslated region of the COX L-type transcripts and that this protein is glutamate dehydrogenase. The mutually exclusive subcellular locations for these mRNAs and glutamate dehydrogenase seem incompatible with an in vivo interaction and consequently with any role in transcript protection or expression. Clearly, the binding species cannot be the precursor form of glutamate dehydrogenase as it is not found in the cytosol (Fig. 1A) and is of identical size to the mature enzyme after denaturing PAGE (Fig. 2C). It is, however, an intriguing observation that, in vitro, transcripts encoding mitochondrially-destined peptides can bind to a protein found within the target organelle. Is it possible that these nuclear-encoded transcripts are accessible to a subset of glutamate dehydrogenase molecules? Previous biochemical and immunohistochemical data, demonstrating two distinct mitochondrial matrix locations for glutamate dehydrogenase, one soluble and one membrane associated, is consistent with this hypothesis [24,25].

To assess this possibility, bovine and rat liver mitochondria were prepared and transcript binding was assayed with intact mitochondria as detailed in section 2.2. RNase T1/heparin-insensitive RNA–protein complexes formed on addition of radiolabelled substrate to the intact organelles, whilst RNase T1 pretreatment of the substrate before mitochondrial addition prevented complex formation (Fig. 3). No increase in complex formation could be achieved by presolubilization of the mitochondria (Fig. 3; cf. conditions c and a). The data shown is representative of numerous experiments with independent mitochondrial preparations. Binding was not lost after solubilization of the outer membrane or by salt washing the organelles (data not shown), indicating that glutamate dehydrogenase liberated from partially lysed mitochondria during homogenisation had not simply become associated with the outer mitochondrial membrane of the intact organelles.

This preliminary data is intriguing. Several recent reports have restated the probability that some mitochondrially-destined polypeptides are cotranslationally translocated [26,27]. Taken in tandem with the effector studies and our recent observation that RNA-binding requires phosphorylation of glutamate dehydrogenase (Preiss et al., manuscript in preparation), it is possible that a subset of transcripts encoding mitochondrially destined polypeptides may be localized to the mitochondrial periphery by a regulated interaction with the RNA-binding domain at membrane contact sites. Once bound, transcripts may be stabilized, and translation products cotranslationally translocated. Alternatively, interactions between glutamate dehydrogenase and its substrate mRNAs may function to keep the polysome anchored to the mitochondrial periphery once the nascent peptide has been fully translocated. This could be of particular importance for short peptides such as the COX subunits which may be not be large enough to maintain polysomal anchoring during cotranslational translocation.

In conclusion, we have demonstrated that the resolvable complexes formed between COX L-type transcripts and cytoplasmic protein all contain the mitochondrial matrix protein glutamate dehydrogenase. The interaction of a nuclear-derived mRNA and a mitochondrial protein may seem paradoxical; however, our preliminary data suggests that the RNA-binding domain of mammalian glutamate dehydrogenase may be accessible to the cytosol. Further experimentation is currently underway to resolve the physiological relevance of this interaction.

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