Subcellular structure containing mRNA for β subunit of mitochondrial H^+ -ATP synthase in rat hepatocytes is translationally active

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We have recently reported that the nuclear-encoded mRNA for the β subunit of mitochondrial H^+ -ATP synthase (β -mRNA) is localized in rounded, electron-dense clusters in the cytoplasm of rat hepatocytes. Clusters of β -mRNA are often found in close proximity to mitochondria. These findings suggested a role for these structures in controlling the cytoplasmic expression and sorting of the encoded mitochondrial precursor. Here we have addressed the question of whether the structures containing β -mRNA are translationally active. For this purpose a combination of high-resolution *in situ* hybridization and immunocytochemical procedures was used. Three different co-localization criteria showed that β -mRNA-containing structures always revealed positive immunoreactive signals for mitochondrial H^+ -ATP synthase (F1-ATPase), ribosomal and hsc70 proteins. Furthermore, clusters show evidence *in situ* of developmental changes

in the translational efficiency of the β -mRNA. These findings suggest that structures containing β -mRNA are translationally active irrespective of their cytoplasmic location. The immunocytochemical quantification of the cytoplasmic presentation of hsc70 in the hepatocyte reveals that approx. 86 % of the protein has a dispersed distribution pattern. However, the remaining hsc70 is presented in clusters of which only half reveal positive hybridization for β -mRNA. The interaction of hsc70 with the β -F1-ATPase precursor protein is documented by the co-localization of F1-ATPase immunoreactive material within cytoplasmic clusters of hsc70 and by the co-immunoprecipitation of hsc70 with the β -subunit precursor from liver post-mitochondrial supernatants. Taken together, these results suggest a role for hsc70 in the translation/sorting pathway of the mammalian precursor of the β -F1-ATPase protein.

INTRODUCTION

Mitochondrial biogenesis results from the co-ordinated expression of the two cellular genomes in which molecular components of the organelle are encoded [1]. Gene activity controls mitochondrial biogenesis through a complex set of mechanisms involving molecules located in different cell compartments [2–5]. Nuclear transcripts encoding mitochondrial proteins are transported to the cytoplasm where they are translated as precursor proteins. Precursors are imported into mitochondria, where they are proteolytically processed to their mature size and often assembled with other proteins, encoded either in the nuclear or mitochondrial genome, to form multimeric complexes [1,6]. Molecular chaperones, both in the cytoplasm and in the mitochondria, are indispensable for biogenesis of mitochondria [7–10].

During certain stages of liver development, the expression of the nuclear-encoded gene for oxidative phosphorylation β -F1-ATPase (the β -subunit of the mitochondrial H^+ -ATP synthase) is exerted at the levels of the stability [11] and translational efficiency [11,12] of the transcript. Concurrently, the control of the expression of the mitochondrial genome is also exerted at the same two post-transcriptional levels of gene expression [13,14]. The operation of these mechanisms of regulation of the expression of the genes involved in organelle biogenesis seems to be a response of a very specific cellular programme of organelle biogenesis, the differentiation of pre-existing mitochondria [11,15]. Differentiation of mitochondria has been assessed both in isolated liver

organelles [11,15,16] or in the liver *in situ* [11], as well as in the regenerating liver [17], in other mammalian tissues [18,19] and in other organisms [20–23].

Recent high-resolution *in situ* hybridization experiments on the β -F1-ATPase mRNA (β -mRNA) in rat hepatocytes have revealed that the cytoplasmic β -mRNA is localized in certain electron-dense structures that are often located in the vicinity of mitochondria [24]. We reported that development does not alter the presentation of β -mRNA in the hepatocytes [24]. However, it has been shown that development does affect the translational efficiency of the transcript [11,12]. These findings suggest that structures containing β -mRNA must represent a specific cytoplasmic location for exerting the regulated translation of the transcript. Furthermore the existence of a localized mRNA in close proximity to mitochondria suggested a role for mRNA localization in targeting the mitochondrial encoded precursor [24].

To address these questions we have combined *in situ* hybridization techniques for β -mRNA and immunoelectron microscopy approaches for F1-ATPase, ribosomal and hsc70 proteins. The results obtained, together with those derived from the cytoplasmic co-localization and co-immunoprecipitation of hsc70 and the β -subunit precursor from rat liver cytosols, strongly suggest that the subcellular site where β -mRNA is confined in the cytoplasm of rat hepatocytes is a translationally active structure. We argue that these findings might have relevant implications for the further characterization of the mechanisms controlling cyto-

Abbreviations used: α - and β -F1-ATPase, subunits α and β of the mitochondrial H^+ -ATP synthase; α - and β -mRNA, α - and β -F1-ATPase mRNA; EM, electron microscopy; mAb, monoclonal antibody.

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plasmic expression and sorting of certain mitochondrial proteins and therefore for the understanding of mammalian mitochondrial biogenesis.

EXPERIMENTAL

Animals

Timed-pregnant albino Wistar rats weighing 200 g were fed with standard laboratory chow and water *ad libitum*. Pregnancy was confirmed on day 19 of gestation. The fetuses (0 h old) (5.2 ± 0.1 g) were delivered by rapid hysterectomy from cervically dislocated pregnant rats on day 22 of gestation. The newborns were maintained without feeding as previously described [15] and killed by decapitation at 1 h after delivery (1 h old). Adult male rats (approx. 250 g) were also used.

Tissue processing for electron microscopy (EM)

Processing of samples for EM was performed as recently reported in detail [24,25]. In brief, small pieces of livers from 0 h, 1 h and adult rats were fixed by immersion in freshly prepared 4% (w/v) paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 M Sörensen phosphate buffer, pH 7.2, for 2 h at 4 °C. After the free aldehyde groups had been quenched [24], tissue samples were processed for embedding in Lowicryl K4M (Chemische Werke Lowi, Waldkraiburg, Germany) in accordance with the manufacturer's instructions. Ultrathin sections (gold interference colour) were collected in Formvar/carbon-coated nickel grids and stored until use. Observation was performed in a Jeol 1010 and a Jeol 1200 ex electron microscope at 80 kV accelerating voltage.

Digoxigenin-labelled cDNA probe

The rat liver β -F1-ATPase cDNA probe [26] was labelled with digoxigenin as recently described in detail [24]. The labelled probe was purified by gel filtration. The probe was precipitated and stored at -70 °C. The labelled probe was resuspended in 10–50 μ l of 10 mM Tris/HCl (pH 8.0)/1 mM EDTA and the amount of digoxigenin incorporated into cDNA was determined by luminescence, following the instructions of the Dig luminescent detection kit (Boehringer Mannheim, Germany).

Simultaneous EM *in situ* hybridization of β -F1-ATPase mRNA and immunocytochemistry of β -F1-ATPase protein, ribosomal phosphoproteins and hsc70

To reveal the subcellular co-distribution of β -mRNA with the β -F1-ATPase precursor protein, ribosomal or hsc70 proteins in the same ultrathin sections, the *in situ* hybridization procedure was performed first followed by protein immunocytochemistry. Briefly, sections were floated on small drops (5 μ l) of the hybridization solution [24,27]. This solution contained 50% (v/v) deionized formamide, 10% (w/v) dextran sulphate, $2 \times$ SSC (SSC is 0.15 M NaCl/0.015 M sodium citrate), 400 μ g/ml salmon sperm DNA and 2.5–16 μ g/ml of digoxigenin-labelled β -F1-ATPase cDNA. Hybridization was performed overnight at 50 °C in a hybridization oven. For details of the immunocytochemical detection of digoxigenin and controls of hybridization experiments the reader is referred to the detailed description in [24]. After the last PBS wash of the *in situ* hybridization protocol (β -mRNA revealed with 10 nm colloidal gold), grids were floated on a drop of 1% glutaraldehyde in PBS for 15 min, rinsed in PBS and incubated with ammonium chloride (50 mM) for 45 min. Grids were then transferred to droplets of anti-(F1-ATPase) serum (1:75 to 1:100) [15], a human antiserum against ribosomal

P_0 , P_1 and P_2 phosphoproteins (1:50) [28,29] or monoclonal antibody (mAb) N27F3-4 to anti-hsc70 (1/75 to 1/100) (StressGene, Victoria, BC, Canada) for 60 min at room temperature. Grids incubated with the polyclonal rabbit [anti-(F1-ATPase complex)] and human [anti-(ribosomal phosphoproteins)] sera were directly transferred to a droplet containing Protein A–gold (15 nm). Grids incubated with mAb (anti-hsc70) were transferred to a droplet of rabbit anti-(mouse IgG + IgM) antibodies and afterwards to the Protein A–gold complex (15 nm). Grids were washed twice in PBS and distilled water, then air-dried. Counterstaining was performed with 2% (w/v) aqueous uranyl acetate (6 min) and 1% (w/v) lead citrate (45 s). In addition to the controls described in hybridization experiments, several controls were conducted in parallel to assess the reliability of the co-localization experiments [24]. These consisted of (1) the omission of the primary antibody against the protein or proteins of interest and (2) the replacement of the labelled cDNA by unlabelled probe.

Simultaneous EM immunocytochemistry of F1-ATPase and hsc70

Nickel grids bearing sections were treated for double immunolabelling as follows: after blocking in 1% (w/v) BSA in PBS, grids were incubated in a droplet of mAb N27F3-4 to anti-hsc70 (1:75 in PBS/1% BSA) (StressGene) for 60 min, rinsed in PBS and incubated for 45 min with goat anti-(mouse immunoglobulins) conjugated with 15 nm colloidal gold (BioCell Research Laboratories, Cardiff, South Glam., U.K.). After incubation, grids were rinsed with PBS and the blocking step was repeated to proceed with incubation with the anti-(F1-ATPase) serum diluted 1:50 in 1% BSA/PBS for 60 min. Grids were further rinsed with PBS and incubated for 45 min in goat anti-(rabbit immunoglobulins) conjugated with 10 nm colloidal gold (BioCell Research Laboratories). After subsequent rinses in PBS and distilled water, grids were stained and observed as previously described. All incubations took place at room temperature. The gold complexes were diluted 1:40 in PBS containing 1% BSA, 0.1% (v/v) Triton X-100 and 0.1% (v/v) Tween-20. As controls for the co-localization experiments, other grids were subjected to the same treatment but incubated with 1% BSA in PBS instead of either the anti-(F1-ATPase) serum or the anti-hsc70 mAb.

Quantification of labelling signals

Quantification of gold particles for the signals revealing β -mRNA hybridization and immunoreactive protein in clusters was performed by two independent investigators directly at the EM screen in a double-blind fashion, using a code that was broken only when the quantification had been finished. The results were expressed as the ratio of gold particles containing mRNA to those containing protein. In hsc70 immunolabelling experiments the results were expressed as the number of gold particles per unit of cellular area. The unit of cellular area was defined as the area of cytoplasm equivalent to approx. 25 μ m² (the micrograph area taken at a magnification of $\times 8000$). To estimate the proportion of hsc70 co-localizing with F1-ATPase in the cytoplasm, the percentage of clusters positive to hsc70 that also presented F1-ATPase labelling was calculated by direct examination in the EM. A total of 100 hsc70-positive clusters were examined.

Immunoprecipitation of pre- β -F1-ATPase and Western blotting of hsc70

The F1-ATPase β -subunit precursor was immunoprecipitated from post-mitochondrial supernatants [24] with 10 μ l of rabbit

Figure 1 β -mRNA clusters reveal differential F1-ATPase immunolabelling during development

In situ hybridization of β -mRNA (10 nm gold) and immunogold labelling for F1-ATPase (15 nm gold) in ultrathin sections of fetal (A) and 1 h postnatal (B) rat liver. β -mRNA and protein co-localize to rounded clusters in the cytoplasm [open arrows in (A) and (B); see also arrowheads in the insets]. Note the presence of F1-ATPase protein labelling in the mitochondrial (mt) cristae and of some F1-ATPase labelling free and scattered in the cytoplasm. Three additional representative hybridization clusters containing gold signals for β -mRNA and protein (arrowheads) in fetal and neonatal hepatocytes are shown in (A) and (B) respectively. Developmental changes in the ratio of β -mRNA hybridization signal to F1-ATPase protein immunocytochemistry are indicated in the text. Abbreviation: N, nucleus. Scale bars: 200 nm in (A) and (B); 150 nm in insets.

anti-(rat liver mitochondrial F1-ATPase) serum [15] essentially as described [16,24]. Briefly, the antigen-antibody reaction was performed for 1 h at 37 °C and overnight at 4 °C in a final volume of 0.8 ml, containing 400 μ l of sample plus 400 μ l of 2 % (v/v) Triton X-100, 500 mM NaCl, 20 mM Tris/HCl, pH 7.5, 1 % (w/v) sodium deoxycholate and 0.2 % SDS. The F1-ATPase antibody complexes were collected by centrifugation at 12 500 *g* for 10 min at 4 °C with the use of Protein A-Sepharose. The immunoprecipitates were washed three times with a medium containing 1 % (v/v) Triton X-100, 250 mM NaCl, 10 mM Tris/HCl, pH 7.5, 0.5 % sodium deoxycholate and 0.1 % SDS. The washed precipitates were fractionated by SDS/PAGE [12.5 % (w/v) gel] and transferred to PVDF membranes [16]. Membranes were incubated with mAb against hsc70 (StressGene). Detection of hsc70 was achieved by membrane incubation with a 1:1500 dilution of rabbit anti-(mouse IgG)-peroxidase conjugate.

Other methods

Fractionation of proteins by SDS/PAGE, electrophoretic transfer of the proteins to PVDF membranes, Western blotting with human polyclonal antiserum against ribosomal phosphoproteins [28] and detection of the immunoreactive proteins, with a goat anti-(human IgG)-peroxidase conjugate (Pierce, Rockford, IL, USA), were performed essentially as described for the β -subunit of the F1-ATPase complex [11,12].

RESULTS

The identification of β -mRNA in a subcellular structure of the rat hepatocyte [24] raised the question of whether such structures are translationally active. To address this question we reasoned that at least three different co-localization criteria must be fulfilled when using a combination of β -mRNA hybridization and immunocytochemistry for different proteins. If structures containing β -mRNA are translationally active, β -mRNA hybridization signals must co-localize first with immunoreactive material revealing the mitochondrial precursor, secondly with ribosomal proteins and thirdly with a cytoplasmic chaperone, such as hsc70, that might be involved in controlling the translation and/or cytoplasmic stabilization of the precursor protein.

Cytoplasmic β -mRNA clusters reveal the presence of F1-ATPase immunoreactive protein

A rabbit polyclonal antibody raised against the native F1-ATPase complex from rat liver [15] was used. It should be emphasized that in addition to the β -subunit, anti-(F1-ATPase) serum also recognizes the α -subunit of the F1-ATPase complex [15]. However, it was used for this purpose because we assumed that co-localization of β -mRNA with F1-ATPase immunoreactive material most probably resulted from recognition of the β -precursor in such structures (a reasonable assumption because

Figure 2 Ribosomal phosphoproteins co-localize with β -mRNA clusters

(A) SDS/PAGE fractionation of 300 μ g of protein from microsomal (lane R) and post-microsomal (lane C) fractions from rat liver Western blotted with human antibodies against the ribosomal P_0 , P_1 and P_2 phosphoproteins. (B) Immunogold labelling (15 nm gold) for ribosomal proteins in ultrathin sections of neonatal rat liver decorates the cytoplasm and the membranes of the endoplasmic reticulum (er). (C) *In situ* hybridization of β -mRNA (10 nm gold) and ribosomal immunocytochemistry (15 nm gold) in neonatal ultrathin sections of rat liver reveal the co-localization of ribosomes within β -mRNA clusters (arrowheads), either when clusters are 'free' in the cytoplasm (C, main panel) or when close to mitochondrial membranes (C, inset). Abbreviation: m, mitochondrion. Scale bars: 500 nm in (B) and (C); 200 nm in inset to (C).

α -mRNA hybridization signals reveal a dispersed non-clustered appearance in rat hepatocytes) [24].

Figure 1 shows representative examples obtained by the combination of both techniques in ultrathin sections of fetal (Figure 1A) and 1 h neonatal (Figure 1B) liver. In agreement with previous findings [24], the results shown in Figure 1 illustrate that development does not alter the subcellular presentation of β -mRNA (small gold particles) in rat liver, i.e. β -mRNA always appears in clusters of hybridization associated with an electron-dense structure of average diameter 150 nm [24]. Gold particles revealing the mitochondrial F1-ATPase complex (large gold particles) decorated the inner mitochondrial membrane. No immunoreactive signals could be observed in the cell nucleus (Figure 1B). Interestingly, F1-ATPase immunoreactive protein was present in cytoplasmic clusters containing β -mRNA hybrids (Figures 1A and 1B, open arrows; see also arrowheads in the

insets), suggesting that clusters are the sites of translation of β -mRNA. It should be noted that some F1-ATPase immunoreactivity was also found free in the cytoplasm of the hepatocyte (Figure 1). Owing to the recognition spectrum of the antibody, it is difficult to know whether such free cytoplasmic F1-ATPase signals also correspond to the β -precursor.

Cytoplasmic β -mRNA clusters reveal differences in the F1-ATPase protein immunoreactive signal as a result of development

It has been documented that the translational efficiency of β -mRNA increases abruptly between the fetal and neonatal stage of liver development [11,12]. Therefore it was interesting to address whether the amount of immunoreactive protein co-localizing in β -mRNA clusters changes during liver development. In such a situation it could provide a further indication that F1-

Figure 3 Molecular chaperone hsc70 is present in cytoplasmic β -mRNA clusters

(A) SDS/PAGE fractionation of 10 μ g of mitochondrial (lane 1) and total (lane 2) rat liver proteins Western blotted with antibodies against hsc70. (B) *In situ* hybridization of β -mRNA (10 nm gold) and hsc70 immunocytochemistry (15 nm gold) in fetal rat liver ultrathin sections; gold particles revealing hsc70 are revealed either free or associated in clusters in the cytoplasm of the hepatocyte. β -mRNA hybridization clusters contain hsc70 protein (open triangle in B). Also, hsc70 immunoreactive clusters with no β -mRNA hybridization signals were observed (solid triangle in B). (C) Quantification of the number of free and clustered gold particles of hsc70 per unit of cytoplasm area (approx. 25 μ m²) reveals that most of hsc70 is distributed in a non-clustered pattern. The results shown in (C) are means $\pm$ S.E.M. ($n = 15$). Abbreviation: m, mitochondrion. Scale bar, 200 nm.

ATPase serum is recognizing the β -subunit precursor within the β -mRNA cluster. A comparison of the hybridization-to-immunoreactivity ratio in cytoplasmic β -mRNA clusters during liver development revealed a significant decrease between fetal and 1 h postnatal samples [means $\pm$ S.E.M., 6.4 ± 0.5 (n , cytoplasmic clusters = 31) and 3.2 ± 0.3 (n , cytoplasmic clusters = 33) for fetal and neonatal ($P < 0.0005$) respectively]. The rapid postnatal change in this ratio is caused by an increase in the protein immunoreactive signal in 1 h-old clusters [compare arrowheads in the inserts below Figure 1(A) with those below Figure 1(B), for fetal and neonatal respectively]. This finding is in agreement with the approx. 2–3-fold increase in translational efficiency experienced by β -mRNA at the time of birth [12]. It should be noted that the increase in the protein immunoreactive signal in cytoplasmic clusters at 1 h postnatal is well above the estimated background of the technique [mean $\pm$ S.E.M., 1.4 ± 0.2 (n , nuclear clusters = 25) protein gold particles per nuclear mRNA hybridization cluster; $P < 0.0005$]. Interestingly, no differences were observed in the hybridization-to-immunoreactivity ratio between 1 h-old postnatal and adult samples (results not shown). Together, the results suggest that the rounded clusters in which β -mRNA is confined in the cytoplasm of rat hepatocytes contain the precursor of the β -subunit of the F1-ATPase complex. Furthermore it seems that the amount of precursor contained in such structures changes in parallel with reported differences in the translational efficiency of the transcript during liver development [12].

β -mRNA clusters co-localize with ribosomal P_0 , P_1 and P_2 phosphoproteins

A polyclonal serum from a patient with systemic lupus erythematosus [28] was used to recognize ribosomal proteins from rat liver. In agreement with previous reports (Figure 2A), the antibodies recognized three phosphoproteins of the 60 S ribosomal subunit (38, 19 and 17 kDa for P_0 , P_1 and P_2 respectively) [28,29]. The specificity of this serum in labelling

ribosomal subunits is illustrated in Figure 2(B). Gold particles decorated the rough endoplasmic reticulum as well as the cytoplasm of the hepatocyte. Other structures such as mitochondria (Figure 2B), peroxisomes or the Golgi stacks (not shown) were not gold-labelled. Co-localization experiments of β -mRNA (small gold particles) with 60 S ribosomal subunits (larger gold particles) revealed that more than 80 % of the cytoplasmic structures containing the β -mRNA contained the large ribosomal subunit (Figure 2C). When the anti-ribosomal antibody was omitted from the procedure, less than 4 % of β -mRNA clusters contained large gold particles, i.e. co-localization is not the result of an artifact of the immunocytochemical procedure (results not shown). It should be stressed that β -mRNA clusters were found to contain the 60 S ribosomal subunit irrespective of its cytoplasmic localization, i.e. either apparently free in the cytoplasm (arrowhead in Figure 2C) or attached to mitochondrial membranes (see inset in Figure 2C), suggesting that β -mRNA clusters are translationally active structures at any location of the cytoplasm where they are present.

Molecular chaperone hsc70 is present in β -mRNA clusters

It has been documented that cytoplasmic molecular chaperones (hsc70, hsp70) interact with the polypeptide chain of mitochondrial precursors to prevent its premature folding before import into mitochondria (reviewed in [30]). Two recent reports have illustrated that hsc70 is required both at the step of processing of the precursor of the β -subunit [31] and at the step of synthesis of the precursor protein of mitochondrial ornithine transcarbamylase [32]. These findings might suggest that hsc70 could also act as a chaperone of the β -F1-ATPase precursor during its synthesis and/or import in rat liver. In line with this idea, if β -mRNA clusters are translationally active structures they should contain the cytoplasmic molecular chaperone hsc70.

Western blots from rat liver homogenates and mitochondrial proteins showed that the antibody against hsc70 was specific for the cytosolic chaperone (Figure 3A, lane 2). In fact, no immuno-

reactivity could be observed in mitochondria (Figure 3A, lane 1), a location where a sequence-related member (grp75) is present [33,34]. *In situ* hybridization of β -mRNA (10 nm gold) in combination with immunocytochemical detection of hsc70 (15 nm gold) revealed that rounded hybridization clusters for β -mRNA were positive for hsc70 (Figure 3B, open triangle). As previously indicated with other antibodies, clusters were gold decorated for hsc70 irrespective of their cytoplasmic location. Hsc70 was evenly located in the cytoplasm (Figure 3B) and nuclei (results not shown) of the hepatocyte. Other subcellular structures such as mitochondria (Figure 3B), peroxisomes, the lumen of the endoplasmic reticulum cisternae and the Golgi stacks showed no gold labelling (results not shown).

Estimation of the number of free and clustered hsc70 signals in the cytoplasm of the hepatocyte (Figure 3C) revealed that approx. 86% of the protein has a dispersed distribution pattern. Furthermore, approx. 50% of the rounded immunoreactive hsc70 clusters were not positive for β -mRNA hybridization (Figure 3B, solid triangle). These findings are in line with the broader function of hsc70 as chaperone of other cellular proteins and suggest that the β -mRNA-negative/hsc70-positive clusters (Figure 3B, solid triangle) could represent specific structures for the location of other mRNA species with similar subcellular distribution patterns to that shown for β -mRNA. These results are the first documentation at the ultrastructural level of the co-localization of the mRNA of a nuclear-encoded mitochondrial protein and the 70 kDa molecular chaperone.

Newly synthesized β -subunit precursor is in close association with hsc70 in cytosol of rat liver

Immunoprecipitation of post-mitochondrial and post-ribosomal supernatants, labelled *in vivo*, from 1 h postnatal liver with anti-(F1-ATPase) serum and further SDS/PAGE fractionation of the resulting immunoprecipitates revealed that most (more than 95%) of the newly synthesized β -subunit precursor is present in post-mitochondrial supernatants [24]. This finding suggested that the β -precursor is not free in the cytosol of rat liver. Two lines of evidence support the suggestion that the β -subunit precursor interacts in the cytosol of rat hepatocytes with hsc70, which suggests a molecular chaperone role of hsc70 for the β -F1-ATPase precursor in rat liver.

First, double immunolabelling experiments of F1-ATPase protein and hsc70 reveal that F1-ATPase immunoreactivity co-localize in cytoplasmic clusters of hsc70 (Figures 4B and 4C). As previously indicated for the co-localization of β -mRNA and hsc70 (Figure 3), only half of the fraction of hsc70 appearing in clusters revealed positive F1-ATPase labelling (approx. 7% of the total hsc70).

Secondly, immunoprecipitation of the β -subunit precursor from post-mitochondrial supernatants obtained from 1 h newborns [24] promotes the co-immunoprecipitation of hsc70, as revealed by Western blotting of the resulting immunoprecipitates (Figure 5, compare lanes 1 and 2). The weak signal of hsc70 that co-immunoprecipitated with the β -F1-ATPase precursor (Figure 5) is in agreement with the small amount of hsc70 estimated to interact with the β -precursor by immunocytochemistry (Figure 4), further supporting the suggestion that only a small fraction of the available cytoplasmic chaperone is involved in the interaction with the β -subunit precursor. This result is in line with a general chaperone role for hsc70 in the biogenesis of cellular proteins [30]. Anyway, these findings further suggest that hsc70 is involved in chaperonin translation/sorting pathway of the mitochondrial precursor of the β -F1-ATPase.

Figure 4 Immunocytochemical procedures reveal the co-localization of hsc70 and F1-ATPase proteins in the cytoplasm of the hepatocyte

Immunocytochemistry of hsc70 (15 nm gold) and F1-ATPase (10 nm gold) in ultrathin sections of rat liver reveal the association of both proteins in cytoplasmic clusters of two different sections [arrowheads in (B) and (C)]. Co-localization of hsc70 and F1-ATPase protein occurred in approx. 50% of hsc70 clusters (see also Figure 3), which represents a small fraction (approx. 7%; see Figure 3C) of the relative cellular availability of hsc70. In (A), a control of the double-immunocytochemical procedure is shown [omission of anti-(F1-ATPase) serum] that illustrates the absence of co-localization of the two sizes of gold particle. Abbreviation: m, mitochondrion. Scale bars, 200 nm.

Fraction of 'active' β -mRNA clusters associated with mitochondria

An estimate of the subcellular localization of 200 clusters of β -mRNA in the cytoplasm of rat hepatocytes revealed that 42 of them (21%) are in close contact with the outer mitochondrial membrane (for illustration of this point, see the micrograph at low magnification in Figure 1A in [24]). It should be noted that

Figure 5 Interaction of the F1-ATPase β -subunit precursor with hsc70 in the cytosol of hepatocytes

Post-mitochondrial supernatants (400 μ l) from 1 h postnatal rat livers were immunoprecipitated with anti-(F1-ATPase) (lane 1) or pre-immune (lane 2) rabbit serum. The antigen-antibody complexes were collected with Protein A-Sepharose, fractionated by SDS-PAGE [12.5% (w/v) gel] and further Western blotted with mAb against hsc70. A rabbit anti-(mouse IgG)-peroxidase conjugate was used as secondary antibody. Note the immunoprecipitation of hsc70 only in lane 1. The rabbit anti-(mouse IgG)-peroxidase conjugate, used as the secondary antibody of the Western procedure, also recognizes the heavy chain of rabbit IgGs used as primary antibodies [see the band above the 46 kDa molecular-mass marker (RPN 756; Amersham), the fourth from the bottom].

this fraction of β -mRNA clusters also showed positive immunoreactive signals for F1-ATPase (Figure 6A, solid arrow), ribosomal phosphoproteins (Figure 2C, insert) and hsc70 (Figure 6B, solid arrow). The β -mRNA clusters found in close proximity to mitochondria suggest that spatial interaction of the structures containing the β -F1-ATPase precursor and mitochondria might

be a limiting factor determining the import of this precursor protein.

DISCUSSION

It has been suggested that localized mRNA species do not become translationally active until placed or anchored in their appropriate subcellular site [35]. For the localized β -mRNA [24] this would imply that cytoplasmic structures containing β -mRNA are not translationally active until they reach the vicinity of mitochondria or are anchored to the outer mitochondrial membrane. Alternatively, and by analogy with the sorting mechanism of proteins destined for the endoplasmic reticulum, there is the possibility that 'free' cytoplasmic clusters of β -mRNA could represent structures containing a translationally arrested state of the transcript and a partly synthesized β -precursor, the translation of the transcript being relieved after placing or anchoring the localized mRNA in close proximity to mitochondria.

The *in situ* hybridization experiments described here provide several lines of evidence supporting the hypothesis that structures containing β -mRNA are translationally active irrespective of their cytoplasmic location (free or attached to mitochondria). In fact we have shown that the electron-dense structures in which β -mRNA is localized in the cytoplasm of rat hepatocytes always contain F1-ATPase, ribosomal and hsc70 proteins (Figures 1, 2 and 3). Furthermore we have shown that the changes in the amount of F1-ATPase immunostaining in β -mRNA-containing structures during liver development (Figure 1) parallel the reported changes in the translational efficiency of β -mRNA during development [12]. These findings are in agreement with those recently reported both by immunoprecipitation and immunolocalization of the β -subunit precursor in the cytoplasm of rat liver [24]. In that study we illustrated the existence of a pool of the full-size newly synthesized β -subunit precursor in the cytoplasm of the hepatocyte, whereas negligible radioactivity was immunoprecipitated in smaller protein fragments [24], i.e. there is no evidence of the cytoplasmic existence of a partly synthesized β -subunit precursor. In any case we should emphasize

Figure 6 Close proximity of a significant fraction of β -mRNA clusters to mitochondrial outer membrane

In situ hybridization of β -mRNA (10 nm gold) and immunocytochemistry (15 nm gold) of F1-ATPase (A) or hsc70 protein (B) in ultrathin sections of fetal rat liver reveal β -mRNA clusters associated with the outer mitochondrial membrane (solid arrows). Large gold particles in (A) reveal the F1-ATPase complex of the inner mitochondrial membrane, whereas large gold particles in (B) reveal hsc70 in cytoplasmic free (open arrow) or membrane-bound (solid arrow) clusters. Scale bars, 200 nm.

that although it is possible to detect the presence of the metabolically labelled β -subunit precursor in rat liver cytosol, no significant accumulation of the precursor was noted [24], suggesting its rapid translocation into the mitochondria.

Although the clustered presentation of β -mRNA is not affected during liver development (Figure 1) [24], the stability [11], translational efficiency [12] and sedimentation behaviour [12] of β -mRNA do experience profound changes as a result of development. The increase in F1-ATPase immunoreactivity observed in β -mRNA clusters in 1 h postnatal liver not only supports the suggestion that these structures are translationally active but also that the regulated translation of β -mRNA during development [12] is exerted within the cluster structures where β -mRNA is confined, in other words that the different physicochemical and biological properties of β -mRNA depend on regulatory proteins that control these activities and are able to interact with these structures in a developmentally regulated manner.

Several hybridization studies *in situ* have shown that certain mRNA species display a patchy pattern in the cytoplasm [36,37], suggesting the association of transcripts with ribonucleoprotein particles. Transport of RNA particles is mediated through its direct or indirect association with cytoskeletal elements [35–37]. The localization of β -mRNA in a particulate structure of the hepatocyte does suggest that clusters containing β -mRNA might be also sorted along the cytoskeleton.

Mitochondrial distribution, translocation and rearrangement within different cellular types have been shown to proceed through the interaction of the organelle with the cytoskeleton of the cell; the physical interaction between mitochondria and the cytoskeleton occurs via actin- or tubulin-associated motor proteins [38–41]. Two different motor proteins for the transport of mitochondria along the actin microfilaments [42] and microtubules [43] have been characterized. Furthermore, recent findings suggest that the sorting of mitochondria within the cytoplasm (cell positioning) and of mitochondrial precursor proteins might share the same cytoskeletal element. In this regard it has been shown that mitochondrial position and inheritance is impaired in yeast mutants bearing conditional lethal mutations in the major actin gene [44,45]. Likewise, genetic evidence in yeast also implicated the actin gene in targeting proteins into mitochondria [46]. The distribution of β -mRNA clusters in the cytoplasm coincides roughly with the distribution of mitochondria within the hepatocyte [24]. Even more, a close spatial association between clusters of β -mRNA and mitochondria has been illustrated (Figures 2 and 6). These findings strongly suggest the existence of a specific site on the mitochondrial surface for the import of the β -precursor (no more than one cluster was found attached per mitochondrial unit). Therefore it is reasonable to suggest that a vectorial sorting pathway of the structure containing β -mRNA along the cytoskeleton, to the site where the mitochondrion is attached to the cytoskeleton, could contribute to increasing the efficiency of delivery of the mitochondrial precursor.

The mechanism of targeting of the precursor protein, based on the localization of the mRNA in the vicinity of mitochondria, prevents the random diffusion of the precursor in the cytoplasm before being recognised by the protein import machinery on the mitochondrial membrane. Localized and bulk import of unique mitochondrial proteins could provide a system with implications for the regulation of mitochondrial biogenesis that deserves to be explored in the future. We should emphasize that the localized mechanism of targeting β -mRNA cannot be generalized to the rest of the nuclear encoded mRNA species of mitochondrial proteins. As recently illustrated, the mRNA encoding the α -F1-

ATPase protein is not localized [24], thus suggesting that the targeting of precursor proteins that follow a cytoplasmic presentation of mRNA similar to that of α -F1-ATPase mRNA might follow a random diffusion mechanism of encounter of the precursor and the protein import machinery on the mitochondrial membrane. In both localized and delocalized targeting pathways of precursor proteins, molecular chaperones and targeting factors [47,48] are expected to participate in assisting and/or specifying the correct targeting of the precursor.

In agreement with the recent role ascribed to hsc70 [31,32], we have shown that hsc70 co-localized in β -mRNA clusters (Figure 3) and is further co-localized (Figure 4) and immunoselected with the β -subunit precursor (Figure 5). These findings strongly suggest that hsc70 is the cytoplasmic chaperone of the β -precursor in mammalian cells. The question now is: what determines the sorting of the structure containing β -mRNA to mitochondrial proximity? It seems that there are at least two different possibilities between which the findings reported here cannot discriminate. First, targeting of β -mRNA clusters to mitochondrial proximity could be mediated by determinants in the β -mRNA sequence. As recently discussed [24], proteins recognizing a specific *cis*-acting element of the β -mRNA sequence, presumably at the 3' untranslated region of the mRNA, could mediate the transport and sorting of the RNA to mitochondrial proximity. However, it should be noted that no such specific β -mRNA-binding protein has been yet characterized in any experimental system. In this case, the localization of the chaperone on the cluster structure would not define its subcellular sorting. However, it is also possible that molecular chaperones and targeting factors recognizing the encoded mitochondrial precursor could specify the targeting of the structures containing β -mRNA to mitochondrial proximity. This latter possibility is compatible with a lack of requirement for a specific cellular set of proteins involved in recognizing elements in the sequence and/or structure of β -mRNA, because this activity could be afforded by the specific set of cytoplasmic proteins involved in the recognition of mitochondrial precursor proteins.

In conclusion, the results presented strongly suggest that clusters are the specific subcellular sites for exerting the regulated translation of β -mRNA and that hsc70 is involved in the translation and/or sorting pathway of the mammalian β -subunit precursor. We hypothesized that import of the β -subunit precursor *in vivo* requires a cell framework to provide the path for sorting the mRNA to mitochondrial proximity, thus preventing the dilution of the β -subunit precursor in the cytoplasm. The above hypothesis is compatible with the identification of an RNA-binding protein capable of rescuing an import defect of the β -subunit precursor [49] and with genetic evidence that implicates the actin cytoskeleton in mitochondrial protein targeting [46].

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