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A 43-kDa Protein Related to c-Erb A α1 Is Located in the Mitochondrial Matrix of Rat Liver*

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In order to characterize Sterling's triiodothyronine (T_3) mitochondrial receptor using photoaffinity labeling, we observed two specific T_3-binding proteins in the inner membrane (28 kDa) and in the matrix (43 kDa) of rat liver mitochondria. Western blots and immunoprecipitation using antibodies raised against the T_3-binding domain of the T_3 nuclear receptor c-Erb A α1 indicated that at least the 43-kDa protein was c-Erb A α1-related. In addition, gel mobility shift assays demonstrated the occurrence of a c-Erb A α1-related mitochondrial protein that specifically binds to a natural or a palindromic thyroid-responsive element. Moreover, this protein specifically binds to a direct repeat 2 sequence located in the D-loop of the mitochondrial genome. Furthermore, electron microscopy studies allowed the direct observation of a c-Erb A-related protein in mitochondria. Lastly, the relative amounts of the 43-kDa protein related to c-Erb A α1 were in good correlation with the known mitochondrial mass in three typical tissues. Interestingly, expression of a truncated form of the c-Erb A α1 nuclear receptor in CV1 cells was associated with a mitochondrial localization and a stimulation of mitochondrial activity. These results supply evidence of the localization of a member of the nuclear receptor superfamily in the mitochondrial matrix involved in the regulation of mitochondrial activity that could act as a mitochondrial T_3-dependent transcription factor.

The regulation of mitochondrial function by thyroid hormone is well documented. Triiodothyronine (T_3)[1] increases the number of mitochondria (Gustafsson *et al.*, 1965; Kadenbach, 1966; Jakovilcic *et al.*, 1978) and the mitochondrial protein synthesis (Mutvei *et al.*, 1989a). Therefore, this hormone is considered to be the major regulator of mammalian mitochondrial biogenesis (Mutvei *et al.*, 1989a). T_3 also stimulates the mitochondrial metabolism (Soboll *et al.*, 1992) and particularly oxidative phosphorylations (Sterling *et al.*, 1977, 1980). More recently, Hafner *et al.* (1990) have shown that thyroid hormone could control state 3 respiration. Some of these effects could involve the activation of mitochondrial gene transcription induced by T_3 (De Leo *et al.*, 1976; Martino *et al.*, 1986; Mutvei *et al.*, 1989b) and the increase in the mRNA of mitochondrial cytochrome *c* oxidase subunit levels (Van Itallie, 1990).

In addition, an early mitochondrial T_3 uptake after [^{125}I]T_3 administration has been reported in electron microscopy studies (Sterling *et al.*, 1984b). In agreement with this observation, evidence of at least one high affinity T_3-binding site was provided by [^{125}I]T_3 binding studies in the mitochondria (Sterling and Milch, 1975; Goglia *et al.*, 1981; Hashizume and Ichikawa, 1982), suggesting that a T_3 mitochondrial receptor (K_a = 10^{11} M^{-1}; molecular mass = 28 kDa) is located in the organelle's inner membrane (Sterling *et al.*, 1978).

However, the involvement of a direct pathway in the mitochondrial action of T_3 is still debated. Therefore, the characterization of the mitochondrial T_3 receptor could be an important tool to verify the existence of such a pathway. The ADP/ATP translocator has been proposed as a putative T_3 mitochondrial receptor (Sterling, 1986). Unfortunately, in agreement with Rasmussen *et al.* (1989), we were unable to observe any T_3 binding activity of this protein, neither in mitochondria nor by testing the purified protein (kindly provided by Dr. Brandolin, Grenoble, France).

Nuclear T_3 receptors are encoded by two different loci leading to the synthesis of three T_3-binding proteins (c-Erb A β1, β2, and α1) sharing homology in the DNA-binding and ligand-binding domains. In addition to the full-length 46-kDa c-Erb A α1 protein, Bigler and Eisenman (1988) have reported the occurrence of several smaller size cellular c-Erb A α proteins in chicken erythroid cells, some of which display an extranuclear location. On the basis of these observations, we searched for the presence of protein(s) related to c-Erb A in mitochondria, and we report here the existence in the mitochondrial matrix of a 43-kDa protein related to c-Erb A α1 with thyroid-responsive element (T_3RE) and T_3 binding activities.

EXPERIMENTAL PROCEDURES

Mitochondria Preparations—Male Wistar rats (body weight, 200 *g*) were injected with Triton WR 1339 (75 mg of Triton/100 *g* of body weight in order to reduce lysosome density) 4 days before euthanasia. Animals were sacrificed after a 16-h period of food deprivation to reduce cellular stocks of lipids and glycogen. Liver mitochondria were prepared by differential centrifugations and purified using a sucrose gradient (1.02/1.68 M) according to Fleischer and Kervina (1974) and Szczesna-Kaczmarek (1990). Mitoplast, inner membrane, outer membrane, and matrix fractions were obtained using digitonin and Lubrol, as described previously by Greenawalt (1974). Lysosome, microsome, plasma

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[1] The abbreviations used are: T_3, triiodothyronine; T_3-PAL, T_3-photoaffinity labeling; T_3RE, thyroid-responsive element; PBS, phosphate-buffered saline; CREB, cAMP-responsive element-binding protein; DR2, direct repeat 2.

membrane, and nuclei fractions were obtained according to Fleischer and Kervina (1974).

The purity of mitochondrial preparations was tested by measuring the specific activities of acid phosphatase (lysosomes; De Duve (1967)), glucose-6-phosphatase (microsomes; Morré (1971)), and 5'-nucleotidase (plasma membranes; Morré (1971)). Monoamine oxidase (outer membrane; Ragan *et al.* (1987)), malate dehydrogenase (matrix; Ragan *et al.* (1987)), and succinate dehydrogenase (inner membrane; Morré (1971)) were also measured to test the submitochondrial fractions (data not shown).

Nuclear contaminations were assessed by Western blots of specific nuclear proteins (lamin A, CREB, and c-Erb A β). Lamin A was detected using an antibody kindly provided by Höger *et al.* (1991) and revealed using a second antibody linked to alkaline phosphatase. c-Erb A β was detected using RHTII antiserum and revealed by ^{125}I-protein A. CREB was detected using anti-rat CREB (rabbit polyclonal antiserum, UBI, Lake Placid, NY) and revealed using the ECL kit (Amersham Corp.).

Electron micrograph was performed on purified rat liver mitochondria. The pellet was fixed by 1% glutaraldehyde/1% paraformaldehyde in phosphate buffer, washed in PBS, postfixed in OsO_4, and embedded in Epon.

Photoaffinity Labeling of T_3-binding Proteins and Western Blots—T_3-binding proteins were detected using a [^{125}I]T_3 photoaffinity label derivative (T_3-PAL), which covalently binds to these proteins after ultraviolet irradiation. [^{125}I]T_3-PAL was synthetized and protein labeling was performed according to Horowitz and Samuels (1988). The 100-μg protein samples were electrophoresed in a SDS-10% polyacrylamide gel (Laemmli, 1970) and autoradiographed. T_3-PAL labeling was performed without and with a previous incubation of mitochondrial proteins with a 1000-fold molar excess of cold T_3-PAL in order to assess labeling specificity.

Western blots of mitochondrial proteins were performed using two different rabbit antisera (IRS 21 and RHTII). IRS 21 is directed against a bacterially expressed protein containing 99 amino acid residues of MS2 polymerase fused to the 96 amino acid residues of the hormone-binding domain of the human c-Erb A α1 nuclear T_3 receptor (Goldberg *et al.*, 1988). RHTII antiserum is raised against the following amino acid sequence: Glu-Cys-Pro-Thr-Glu-Leu-Phe-Pro-Pro-Leu-Phe-Leu-Glu-Val-Phe-Glu (399–414 of c-Erb A α1 xenopus receptor, 392–407 of c-Erb A α1 chicken receptor, 391–406 of c-Erb A α1 rat receptor, and 440–455 of c-Erb A β rat receptor).

Immunoprecipitation and Gel Mobility Shift Assays—Immunoprecipitation of [^{125}I]T_3-PAL-binding proteins was performed with IRS 21 antiserum (Sambrook *et al.*, 1989).

Prior to gel mobility shift assays, purified mitochondria were lysed at 4 °C for 60 min with two volumes of solution A (10 mM Hepes, pH 7.9, 0.4 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol, 0.5% Nonidet P40). After centrifugation of the lysate (130,000 × *g* for 60 min), the supernatant was dialyzed in solution B (20 mM Hepes, pH 7.9, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol) with 50 mM KCl and was loaded onto a heparin-agarose column equilibrated in solution B/50 mM KCl. The column was washed with solution B/50 mM KCl and eluted with solution B/300 mM KCl. Fractions containing proteins were pooled and concentrated by precipitation with ammonium sulfate. After centrifugation, the pellet was suspended and then dialyzed in solution B/50 mM KCl.

Gel mobility shift assays were performed according to Graupner *et al.* (1989) using a ^{32}P-labeled palindromic T_3RE as a probe (GATCCTCAGGTCATGACCTGAA). Specificity of DNA binding was tested by competition with 200 ng of cold palindromic T_3RE or with a similar amount of a mutated carbonic anhydrase II T_3RE unable to bind nuclear T_3 receptors (Pal-I: GATCCGAGTGGTGATTCAACTGCTA). A natural T_3RE (Pal-2) identified on the upstream sequence (−669/−650) of the anhydrase carbonic II gene (Disela *et al.*, 1991) was also tested.

Similar experiments were performed using a synthetic oligonucleotide (TAGCCGTCAAGGCATGAAGGTCAGCAC) corresponding to a direct repeat sequence (DR2) (Bogazzi *et al.*, 1994) observed in the rat D-loop (15923–15949) according to the complete nucleotide sequence of the *Rattus norvegicus* mitochondrial genome (Gadaleta *et al.*, 1989).

Electron Microscopy—Electron microscopy was performed on Wistar rat liver preparations (cryoultramicrotomy and immunolabeling). Specimens were fixed with 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h at 4 °C. The fixed specimens were infused with 2.3 M sucrose, rapidly frozen. Ultrathin frozen sections (80 nm) were prepared and transferred onto Formvar films on electron microscope grids. They were washed with 0.1 M phosphate buffer, pH 7.4, containing 0.65 M NaCl, 0.05% Tween 20, and 0.5% ovalbumin and then incubated with RHTII antiserum (diluted 1/1000 in PBS) for 45 min. After the washes, the sections were incubated for 45 min with goat anti-rabbit antiserum diluted 1/25 in 20 mM Tris buffer, pH 7.6, containing 0.65 M NaCl, 0.05% Tween 20, and 0.5% ovalbumin. Ultrathin sections were then osmicated, dehydrated, stained with 1% aqueous uranyl acetate, and examined in a Zeiss EM902 electron microscope.

Cytoimmunofluorescence Study—Simian CV1 cells (ATCC CCL 70) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were carefully washed in PBS and fixed for 10 min in freshly prepared 3% (w/v) paraformaldehyde with 0.05% Tween 20. The cells were again washed in PBS and incubated in methanol for 10 min at −20 °C. Cells were then incubated in 50 mM glycine for 5 min and washed in PBS. Fixed cells were blocked for 20 min in normal goat serum and incubated in a mixture of rabbit RHTII antiserum (final dilution 1/100) and monoclonal antibody against mitochondria (anti-mitok, Chemicon International, Inc.; final dilution, 1/30) in PBS plus 0.5% bovine serum albumin for 1 h. After washing for 3 × 5 min in PBS with 0.1% Tween 20, cells were incubated with a rhodamine-conjugated goat anti-mouse antibody and a fluorescein-conjugated goat anti-rabbit antibody. After a final 3 × 5 min wash in PBS with 0.1% Tween 20, culture dishes were mounted and photographed using a standard Zeiss Axiophot immunofluorescence microscope.

Expression of a Truncated c-Erb A α1 Receptor—CV1 cells stably expressing truncated Erb A protein were obtained by transfecting plasmid pF1Δmet1 (kindly provided by Bigler and Eisenman; Bigler *et al.* (1992)). One day prior to transfection, the cells were plated at 5 × 10^5 cells/10-cm dish. DNA was transfected into those cells by the $Ca_3(PO_4)_2$ coprecipitation method with pSV-neo as a selectable marker. Individual clones were grown in the presence of G418 (800 μg/ml).

Assessment of Mitochondrial Activity—Mitochondrial activity was observed in living cells by measurement of rhodamine 123 uptake (Johnson *et al.*, 1980) as a marker of mitochondrial membrane potential (Chen, 1988) and of cytochrome oxidase activity (Wharton and Tzagaloff, 1967).

RESULTS

Assessment of the Purity of Mitochondrial Preparations—Electron microscopy examination did not demonstrate the occurrence of extramitochondrial components in our preparations (Fig. 1*A*). Measurements of specific activities of acid phosphatase, glucose-6-phosphatase, and 5'-nucleotidase demonstrate that contaminations by lysosome, microsome, and membrane proteins were always minimal (5.2, 9.8, and 4.3%, respectively; Fig. 1*B*).

Moreover, a nucleus-specific protein, lamin A, was not detected by Western blot in 100 and 200 μg of mitochondrial proteins, whereas it was clearly apparent in 10 μg of nuclear proteins (Fig. 1*C*). Similarly, the nuclear cAMP-dependent transcription factor CREB was easily detected in 10 μg of nuclear proteins and was on the brink of detection in 5 μg of nuclear proteins; however, it was not detected in 400 and 200 μg of mitochondrial proteins (Fig. 1*D*). In addition, although RHTII antiserum recognizes the c-Erb A α1 and β forms of T_3 nuclear receptors, in our mitochondrial preparations, we were unable to detect c-Erb A β, the major isoform in liver nuclei (Fig. 1*E*).

Detection of T_3 Binding and Proteins Related to c-Erb A α1 in Rat Liver Mitochondria—We searched for T_3-binding proteins in purified mitochondrial preparations from rat liver. In all samples, three proteins were identified by [^{125}I]T_3-PAL labeling (Fig. 2*A*) with apparent molecular mass in SDS-polyacrylamide gel electrophoresis of approximately 43, 41, and 28 kDa. In agreement with the data of Rasmussen *et al.* (1989), a T_3-PAL signal could not be detected in the 30-kDa range, indicating that the ADP/ATP translocator is unlikely to bind T_3 significantly. Furthermore, a purified preparation of the ADP/ATP translocator could not be labeled after incubation in the presence of [^{125}I]T_3-PAL (data not shown). Labeling of the 43-, 41-, and 28-kDa proteins is specific, because it is competed with a 1000-fold excess of unlabeled T_3-PAL (Fig. 2*A*).

Similarly sized proteins (43, 41, and 28 kDa) were observed by immunoblotting analysis of mitochondrial extracts using

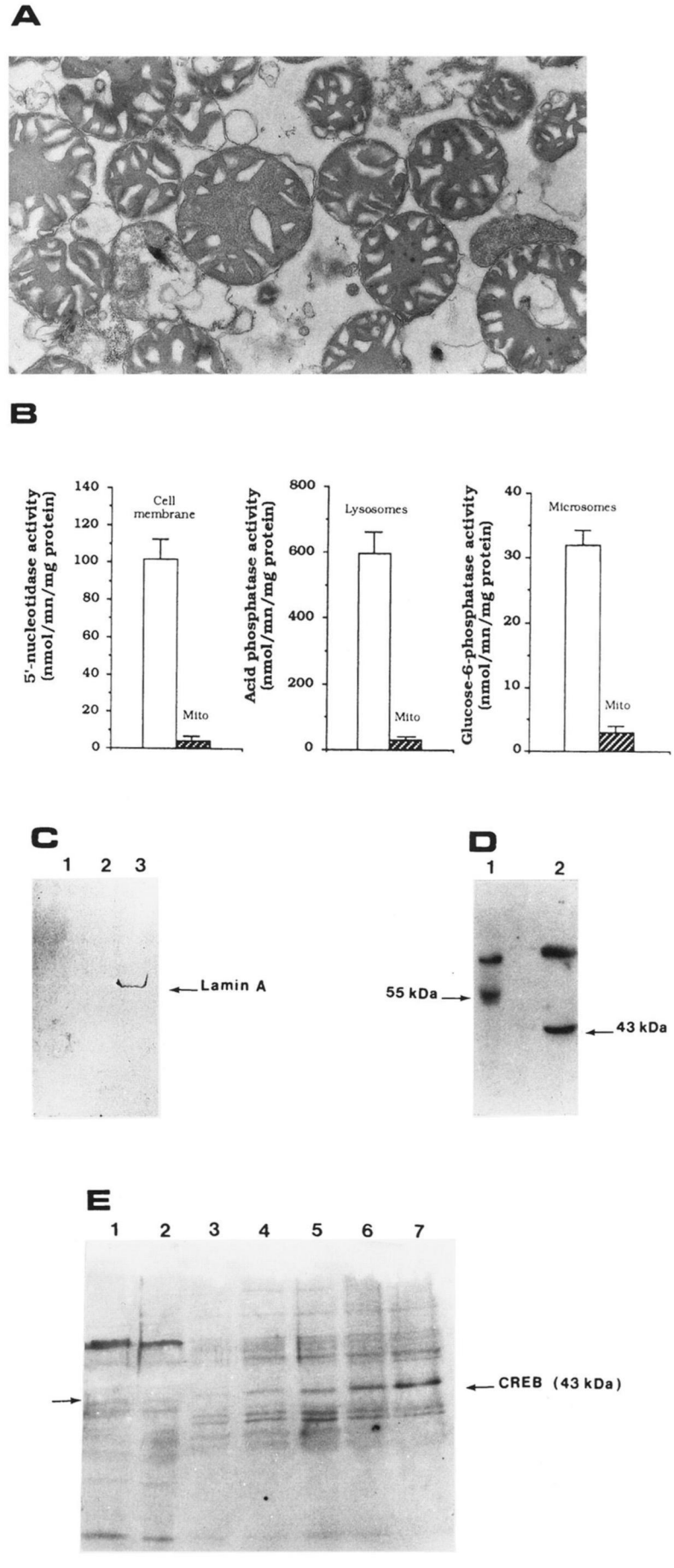

FIG. 1. **Assessment of the purity of mitochondrial preparations.** *A*, electron micrograph of purified rat liver mitochondria. All the recognizable organelles are mitochondria showing different degrees of density of the matrix and of dilatation of the cristae (×18,000). *B*, specific activities of acid phosphatase (lysosomes), glucose-6-phosphatase (microsomes), and 5′-nucleotidase (plasma membranes) in mitochondrial (*Mito*) preparations. Data are presented as the means of 10 different experiments. *C*, Western blot of nuclei and mitochondrial preparations using an antibody raised against a specific nuclear protein, lamin A. *Lane 1*, 100 μg of mitochondrial protein; *lane 2*, 200 μg of mitochondrial protein; *lane 3*, 10 μg of nuclear protein. *D*, Western blot of nuclei and mitochondrial preparations using RHTII antiserum. *Lane 1*, 50 μg of nuclear protein; *lane 2*, 50 μg of mitochondrial protein. *E*, Western blot of nuclei and mitochondrial preparations using an antiserum raised against rat CREB. *Lane 1*, 400 μg of mitochondrial protein; *lane 2*, 200 μg of mitochondrial protein; *lane 3*, 5 μg of nuclear protein; *lane 4*, 10 μg of nuclear protein; *lane 5*, 20 μg of nuclear protein; *lane 6*, 50 μg of nuclear protein; *lane 7*, 100 μg of nuclear protein. In each case, mitochondrial and nuclei preparations were obtained from the same fresh sample of rat liver.

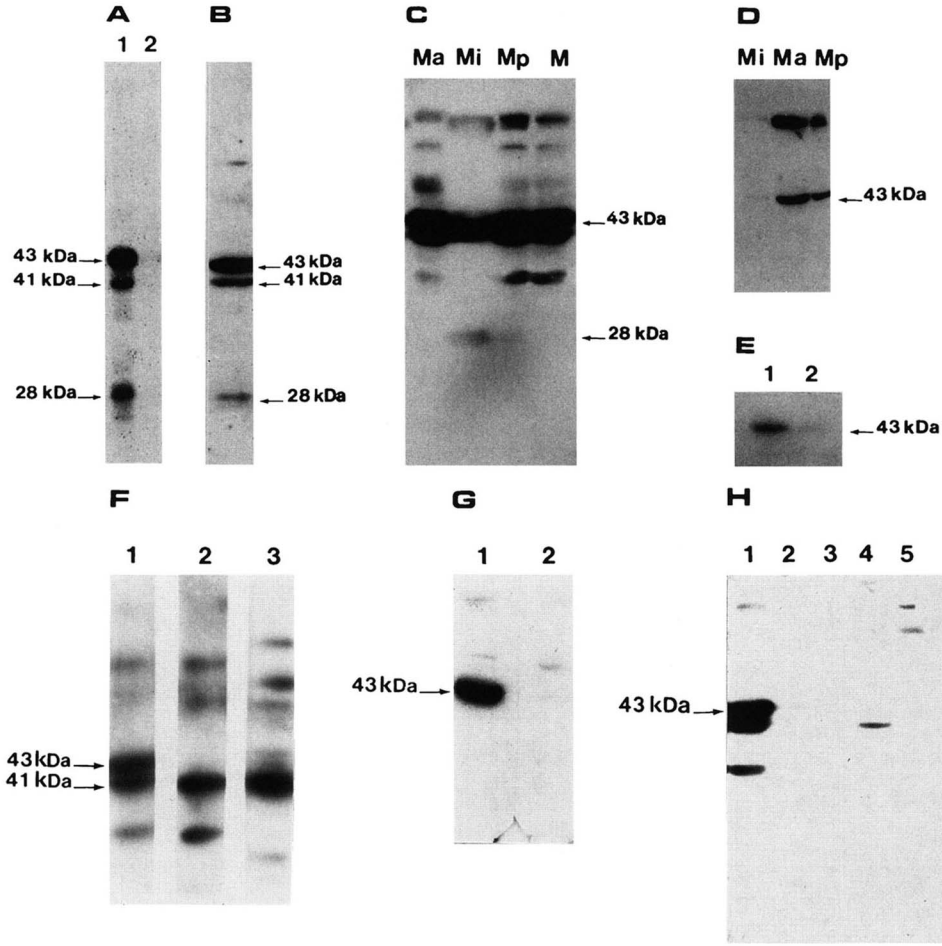

FIG. 2. **Detection of T_3 binding and c-Erb A α1 proteins in rat liver mitochondria.** *A*, photoaffinity labeling (T_3-PAL) of 100 μg mitochondrial proteins. T_3-PAL labeling was performed without (*lane 1*) and with (*lane 2*) a previous incubation of mitochondrial proteins with a 1000-fold molar excess of cold T_3-PAL in order to assess labeling specificity. *B*, Western blot of mitochondrial proteins using IRS 21 antibody (100 μg of protein/well). *C*, intramitochondrial localization of c-Erb A α1-related protein shown on Western blot using IRS 21 antiserum. *M*, purified mitochondria, *Mp*, mitoplast fraction, *Mi*, inner membranes; *Ma*, matrix fraction. *D*, Western blot of mitoplast (*Mp*), matrix (*Ma*), and inner membrane (*Mi*) proteins using RHTII antiserum (100 μg of protein/well). *E*, immunoprecipitation of a [^{125}I]T_3-PAL-labeled protein by IRS 21 antiserum. *Lane 1*, IRS 21 alone; *lane 2*, IRS 21 previously incubated with a large excess of MS2-Erb A α protein. *F*, the 43-kDa protein is specifically detected by IRS 21 antiserum in purified mitochondria (100 μg of protein/well). *Lane 1*, IRS 21 antiserum; *lane 2*, MS2/c-Erb A peptide preabsorbed IRS 21 antiserum; *lane 3*, MS2 antiserum. *G*, RHTII antiserum also specifically detects a 43-kDa protein in purified mitochondria (100 μg of protein/well). *Lane 1*, RHTII antiserum; *lane 2*, preimmune RHTII serum. *H*, specific mitochondrial localization of the 43-kDa c-Erb A-related protein. Western blots using IRS 21 antiserum were performed on 50-μg protein samples. *Lane 1*, mitochondria; *lane 2*, lysosomes; *lane 3*, plasma membranes; *lane 4*, microsomes; *lane 5*, nuclei.

IRS 21, a specific anti-Erb A α antibody (Fig. 2*B*). IRS 21 is directed against a bacterially expressed MS2 polymerase/c-Erb A α fusion protein. Blockade of c-Erb A α IRS 21 antibody with its nominal antigen or the use of an antibody directed against the MS2-encoded domain of the fusion protein indicated that antibody binding to the 41-kDa protein was not specific (Fig. 2*F*). In addition, Western blot performed with RHTII antiserum only specifically detected a 43-kDa protein in mitochondrial preparations (Fig. 2*G*).

Following mitochondrial subfractionation, we showed that 43 and-28-kDa proteins were detected by T_3-PAL labeling (data not shown) and Western blots (IRS 21, Fig. 2*C*) in the mitoplast fraction. Whereas the 28-kDa protein was for the most part localized in the inner membrane, the 43-kDa protein showed a major localization in the matrix, although lower relative amounts could be detected in the inner membrane fraction. Interestingly, Western blots performed with RHTII antiserum also detected a 43-kDa protein in the mitochondrial matrix (Fig. 2*D*). As verified by Western blots (IRS 21), no signal corresponding to the 43-kDa protein appeared on lysosome, microsome, membrane, and nuclear preparations (Fig. 2*H*), demonstrating a specific mitochondrial localization.

Using IRS 21 antibody and T_3-PAL-labeled mitochondrial preparations, we were able to immunoprecipitate a 43-kDa [^{125}I]T_3-PAL-labeled protein. This did not significantly occur when the antibody was preincubated with an excess of the MS2/Erb A α protein (Fig. 2*E*). Therefore, the 43-kDa T_3-binding protein was identified to be immunologically related to the 43-kDa protein detected by Western blot. In agreement with this set of data, using RHTII antibody in electron microscopy studies, we have directly observed a specific liver mitochondrial labeling, which does not appear when using the preimmune serum or the c-Erb A preabsorbed antiserum (Fig. 3).

The Mitochondrial 43-kDa Protein Related to c-Erb A α1 Specifically Binds to a T_3-responsive Element and a DR2 Sequence Located in the Mitochondrial D-loop—In order to further extend similarities between the mitochondrial 43-kDa protein and the c-Erb A α1 nuclear receptor, we analyzed the DNA binding properties of the mitochondrial protein. The 43-kDa [^{125}I]T_3-PAL-labeled mitochondrial protein bound to DNA cellulose and to heparin-agarose columns (data not shown). Gel mobility shift assays performed with mitochondrial preparations partially purified on heparin-agarose showed that a protein or a protein complex binds to a palindromic T_3RE (Fig. 4,

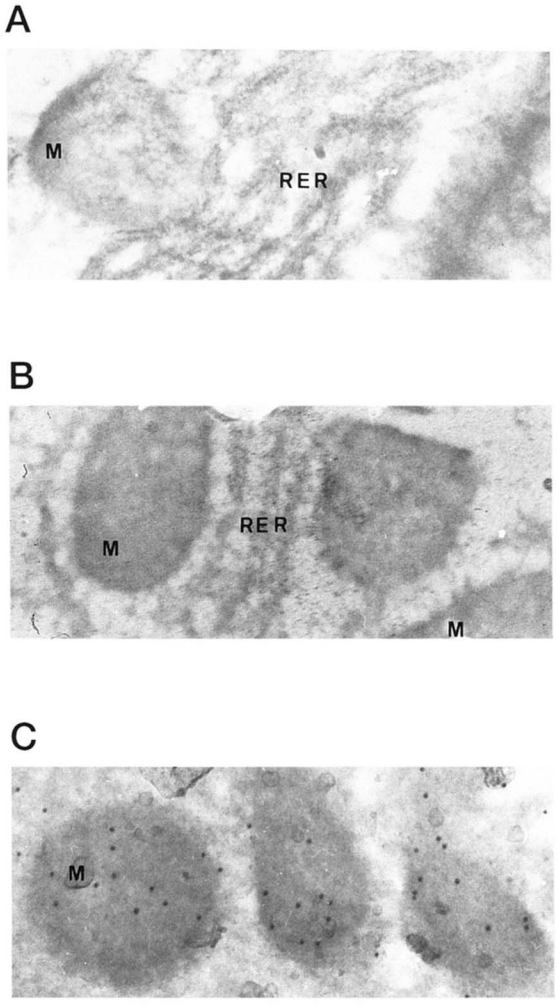

FIG. 3. **Observation of a protein related to c-Erb A in rat liver mitochondria by electron microscopy (×42,000).** *M*, mitochondria; *RER*, rough endoplasmic reticulum. *A*, preimmune RHTII serum. *B*, c-Erb A peptide preabsorbed RHTII antiserum. *C*, RHTII antiserum.

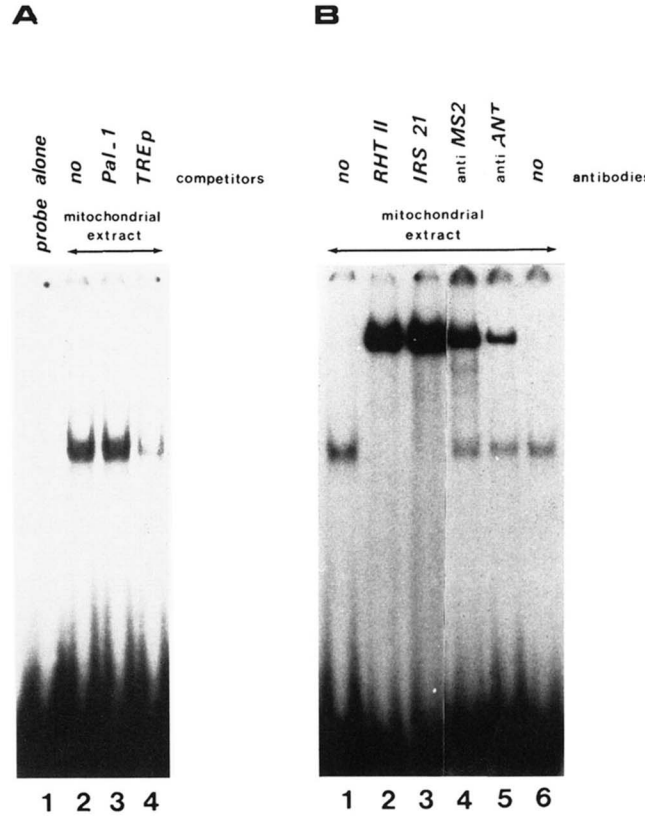

FIG. 4. **A mitochondrial protein partly purified on heparin-agarose specifically binds to a palindromic T_3RE.** *A*, gel mobility shift assays (2 μg of mitochondrial protein). *Lane 1*, labeled alone; *lane 2*, labeled T_3RE and mitochondrial extract; *lane 3*, competition with 200 ng of a cold mutated T_3RE (*Pal-1*); *lane 4*, competition with 200 ng of cold T_3RE (*TREp*). *B*, specific recognition by c-Erb A antisera of the mitochondrial T_3RE-binding protein. *Lanes 1* and *6*, mitochondrial extracts. Mitochondrial extracts were preincubated with RHTII (*lane 2*), IRS 21 (*lane 3*), MS2 antiserum (*lane 4*), or rat ADP/ATP translocator (*lane 5*) antiserum.

A and *B*) and a natural T_3RE identified on the chicken carbonic anhydrase II gene (data not shown). An excess of cold T_3RE was found to compete binding to the probe, whereas a similar molar excess of a mutated T_3RE unable to bind T_3 receptors did not, thus demonstrating specificity of the binding (Fig. 4*A*). Moreover, in contrast to nonspecific antisera (raised against MS2 and rat ADP/ATP translocator), IRS 21 and RHTII antisera suppressed the signal observed for mitochondrial extracts (Fig. 4*B*). Because RHTII antiserum only detects the 43-kDa protein in heparin-agarose purified mitochondrial extracts, these results demonstrate that the 43-kDa protein also shares strong homology in the DNA-binding domain with the nuclear T_3 receptors.

Interestingly, a similar retardation band was observed after interaction of heparin-agarose-purified mitochondrial extracts with a DR2 sequence located in the D-loop of the rat mitochondrial genome (Fig. 5). This result clearly indicated that the 43-kDa protein binds to a specific sequence residing in the D-loop area, which contains the promotors and is not transcribed.

The 43-kDa Protein Related to c-Erb A α1 Is a Possible T_3 Mitochondrial Receptor—Because Sterling *et al.* (1977) reported the absence of mitochondrial T_3 receptors in adult rat brain, we performed photoaffinity labeling and Western blots of rat brain mitochondrial proteins. As shown in Fig. 6*A*, the 43-kDa protein was not detected in these preparations.

We have compared the amounts of the 43-kDa protein in mitochondria obtained from three tissues showing great differences in mitochondrial mass: brown adipose tissue, liver, and white adipose tissue in decreasing order. This study was performed using purified mitochondria from 12-day-old rabbits, in which brown and white adipose tissues are easily collected simultaneously. We showed that the relative mitochondrial amounts of the 43-kDa protein are higher in brown adipose tissue than in liver and are higher in liver than in white adipose tissue (Fig. 6*B*). Therefore, a clear relationship exists between the mitochondrial mass and the amount of the 43-kDa protein in the mitochondrion, thus suggesting an important mitochondrial function for this protein.

Because the 43-kDa protein shared strong homology in the DNA and T_3-binding domains with the nuclear T_3 receptors, the lower molecular weight of the mitochondrial protein could be explained by deletion of the hinge region or of the N-terminal domain of c-Erb A α1 receptor. However, Lin *et al.* (1992) have reported that deletion of the hinge region abolished T_3 binding activity of the protein. Therefore, using the pF1Δmet1 plasmid, we overexpressed in CV1 cells a truncated c-Erb A α1 protein lacking the N-terminal domain (A/B domains) of the nuclear receptor. Interestingly, we observed a strong increase in mitochondrial staining by RHTII antibody when compared with control cells (transfected with the empty vector, pEMSV; Fig. 7). This result strongly suggested that the overexpressed protein displayed a major mitochondrial location.

Lastly, overexpression of the truncated protein induced a

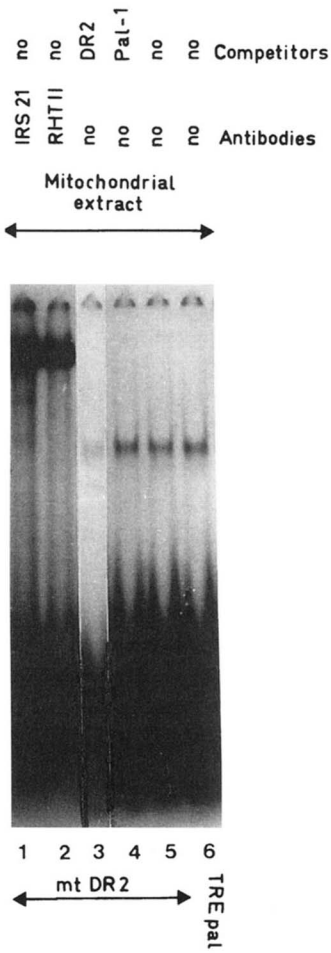

FIG. 5. **The mitochondrial protein related to c-Erb A binds to a specific DR2 sequence located on the D-loop of mitochondrial genome.** Gel mobility shift assays (2 μg of mitochondrial protein) are shown. *Lanes 1–5*, DR2 probe. Mitochondrial extracts were preincubated with IRS21 (*lane 1*) or RHTII (*lane 2*) antisera. *Lane 3*, competition with 200 ng of cold DR2; *lane 4*, competition with 200 ng of cold mutated T_3RE (*Pal-1*); *lane 6*, mitochondrial extract and labeled palindromic T_3RE (*TREpal*).

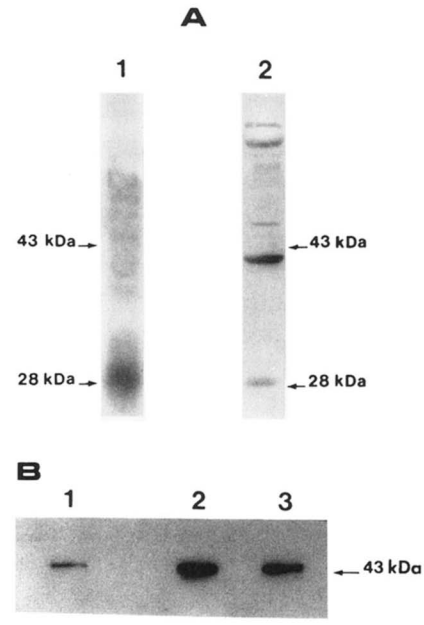

FIG. 6. **The mitochondrial amounts of the 43-kDa protein related to c-Erb A α1 display cell specificity.** *A*, adult rat brain mitochondria (100 μg of protein/well). *Lane 1*, T_3-PAL labeling; *lane 2*, Western blot using RHTII antiserum. *B*, relative amounts of the 43-kDa c-Erb A α1 protein in mitochondrial extracts (30 μg of protein) of white adipose tissue (*lane 1*), brown adipose tissue (*lane 2*), or liver (*lane 3*).

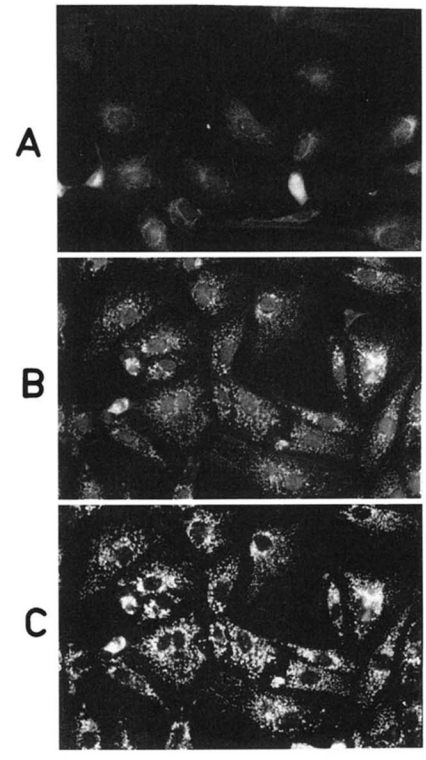

FIG. 7. **An overexpressed truncated c-Erb A α1 protein displays a mitochondrial location in CV1 cells.** Cytoimmunofluorescence studies (×400) are shown. *A*, staining of control CV1 cells by RHTII antibody. *B*, staining of CV1 cells overexpressing the c-Erb A truncated protein by RHTII antibody. *C*, same field as in *B* labeled with a monoclonal antibody against mitochondria (anti-mitok).

strong stimulation of rhodamine 123 uptake (Fig. 8*A*) and cytochrome oxidase activity (Fig. 8*B*). All these data clearly indicated that a mitochondrial truncated c-Erb A α1 protein strongly affects the organelle function.

DISCUSSION

Our data recording mitochondrial T_3-binding proteins confirm the results of the Sterling group, who reported the presence of a 28-kDa T_3-binding protein in the mitochondrial inner membrane. In addition, we show that a 43-kDa T_3-binding protein is present in the mitochondrial matrix. This binding site was not observed by the Sterling group, probably because purification steps leading to the concept of a 28-kDa mitochondrial receptor were performed using inner membrane preparations after discarding matrix proteins (Sterling *et al.*, 1984a). Furthermore, we report here that at least one mitochondrial T_3-binding protein is related to c-Erb A-encoded nuclear receptors.

Among these T_3-binding proteins, we also observed a 41-kDa protein obviously not related to c-Erb A nuclear receptors. We observed by T_3-PAL binding (data not shown) that it was also detected in microsome, plasma membrane, and lysosome preparations, thus ruling out a specific mitochondrial localization. Our results concerning the 28-kDa T_3-binding protein are not fully conclusive; the recognition specificity by IRS 21 remains unclear, and we were unable to immunoprecipitate this protein by IRS 21 and to detect it by RHTII antiserum. However, these negative data could be explained by the very low amounts of the 28-kDa protein recorded into mitochondria. By contrast, the mitochondrial 43-kDa protein is clearly related to the c-Erb

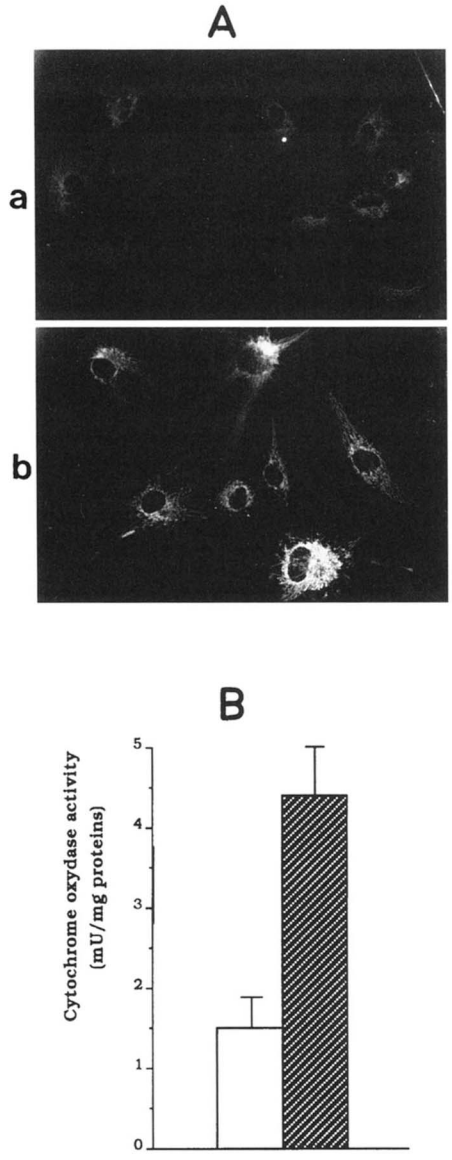

FIG. 8. **An overexpressed truncated c-Erb A α1 protein stimulates mitochondrial activity.** *A*, living cells stained with rhodamine 123. *a*, control CV1; *b*, pF1Δmet1-transfected CV1 cells. *B*, cytochrome oxidase activity in control (*open bar*) and truncated c-Erb A expressing CV1 cells (*hatched bar*)

A α1 nuclear receptor. Antisera raised against two different amino acid sequences of the T_3-binding domain of c-Erb A specifically detect this protein in the matrix. Moreover, a 43-kDa T_3-binding protein is specifically immunoprecipitated by IRS 21 antiserum. Lastly, gel retardation experiments demonstrate that this mitochondrial protein binds to T_3REs DNA sequences. This set of data strongly suggests that this protein shares homology in the DNA binding and T_3-binding domains with c-Erb A nuclear receptors.

The identification of a protein related to c-Erb A α1 in the mitochondrion is not the consequence of contamination of our preparations by nuclei or components of other cell compartments because (*a*) Contaminations by lysosome, microsome, and membrane proteins were always minimal, as verified by measurements of specific markers. (*b*) Nucleus-specific proteins lamin A and CREB were not detected in our mitochondrial preparations. (*c*) RHTII antibody recognizes c-Erb A α1 and β forms; although the β form is at least four times more abundant than the α1 form in rat liver nuclei (Schwartz *et al.*, 1992; Rodd *et al.*, 1992), we were unable to detect c-Erb A β in our mitochondrial preparations. If the localization of a c-Erb A protein into the mitochondrion was the result of nuclear contaminations, a major β form would be detected. (*d*) As verified by Western blot, the signal corresponding to the 43-kDa protein appeared only in mitochondrial preparations. (*e*) Treatment of mitochondria with trypsin did not affect the 43-kDa signal in Western blot experiments (data not shown). (*f*) *In situ* electron microscopy studies bring direct evidence of the presence of a protein related to c-Erb A in the mitochondrion. Indeed, the 43-kDa protein related to c-Erb A α1 displays a specific mitochondrial localization.

Interestingly, we observed that the T_3-binding 43-kDa protein related to c-Erb A α1 was not detectable in adult rat brain mitochondria, in agreement with the previously reported lack of T_3 receptors in these mitochondria (Sterling *et al.*, 1977). In addition, the positive relation recorded between the amounts of this protein into the organelle and the mitochondrial mass of three characteristic tissues strongly suggests a possible involvement of the 43-kDa protein in the regulation of mitochondriogenesis, a T_3-regulated process (Gustafsson *et al.*, 1965; Kadenbach, 1966; Jakovilcic *et al.*, 1978).

All these data strongly suggest that the 43-kDa protein is a putative T_3 mitochondrial receptor. This hypothesis is well supported by the additional observation that overexpression of a truncated c-Erb A α1 protein displaying a mitochondrial localization induced a strong stimulation of the organelle activity assessed by rhodamine 123 uptake and cytochrome oxidase activity, a well known target of T_3 influence at the mitochondrial level.

The origin and function of this protein is finally questioned. Taking into account the sequence of the mitochondrial genome in several species, the 43-kDa protein related to c-Erb A α1 is clearly encoded by a nuclear gene and is translocated into the mitochondrion.

To date, five different c-*erb* A mRNAs have been identified in rats, encoding c-Erb A β1, β2, α1, α2, and α3 (v II) proteins. Despite intensive studies, no additional mRNAs have been identified that could lead to the specific synthesis of a 43-kDa c-Erb A α1 protein. Using polymerase chain reaction in rat liver mRNAs preparations, we were also unable to detect c-*erb* A α1 mRNAs other than that encoding the 46-kDa protein (data not shown). Nevertheless, the possibility of such a mRNA could not be excluded.

Another possibility is raised by the observation of Sap *et al.* (1986) that *in vitro* translation of a chicken c-*erb* A α1 mRNA gives rise to two major proteins with molecular mass of 46 kDa (as expected) and approximately 40 kDa. The same was observed by these workers after transfection of the c-*erb* A α1 cDNA in chicken embryo fibroblasts. Therefore, the c-*erb* A α1 mRNA can encode two proteins. Additional support is supplied by the identification of several c-Erb A α1 proteins in cells (Bigler and Eisenman, 1988), and the observation that some of them have an extranuclear localization. In addition, Bigler *et al.* (1992) reported that the smaller receptor forms are generated by alternative translational initiations at internal AUGs in full-length *erb* A α1 mRNA. Therefore, we suggest that the mitochondrial 43 kDa T_3-binding protein could be the product of the mRNA c-*erb* A α1 using an internal AUG. In agreement with this hypothesis, we observed that overexpression of a truncated c-Erb A α1 protein corresponding to the product obtained by utilization of the first internal AUG lead to the synthesis of a mitochondrial protein inducing a stimulation of mitochondrial activity.

Interestingly, the 43-kDa T_3-binding protein is localized in the mitochondrial matrix and thus is in physical contact with mitochondrial DNA. Several studies reported that T_3 stimu-

lates mitochondrial gene transcription (De Leo *et al.*, 1976; Martino *et al.*, 1986; Mutvei *et al.*, 1989b). We have shown that this 43-kDa protein is strongly related to the c-Erb A α1 nuclear receptor, a well known T_3-dependent transcription factor. This protein displayed a sequence-specific DNA binding activity and particularly is able to bind to a specific sequence of the mitochondrial D-loop. Therefore, it could be proposed that the 43-kDa T_3-binding protein could be the first hormone-dependent transcription factor identified in the mitochondrion.

In conclusion, our work suggests the presence of a T_3 mitochondrial receptor, which has the truncated form of the T_3 nuclear receptor c-Erb A α1 in the mitochondrial matrix, which could act as a T_3-dependent transcription factor. This hypothesis could explain the specific action of thyroid hormone on the mitochondria, particularly the stimulation of mitochondrial gene transcription induced by T_3.

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REFERENCES

Bigler, J., and Eisenman, R. N. (1988) *Mol. Cell. Biol.* **8,** 4155–4161
Bigler, J., Hokanson, W., and Eisenman, R. N. (1992) *Mol. Cell. Biol.* **12,** 2406–2417
Bogazzi, F., Hudson, L. D., and Nikodem, V. M. (1994) *J. Biol. Chem.* **269,** 11683–11686
Chen, L. B. (1988) *Annu. Rev. Cell Biol.* **4,** 155–181
De Duve, C. (1967) *Methods Enzymol.* **10,** 7–18
De Leo, T., Di Meo, S., Barletta, A., Martino, G., and Goglia, F. (1976) *Pfluegers Arch. Eur. J. Physiol.* **366,** 73–77
Disela, C., Glineur, C., Bugge, T., Sap, J., Stengl, G., Dodgson, J., Stunnenberg, H., Beug, H., and Zenke, M. (1991) *Genes & Dev.* **5,** 2033–2047
Fleischer, S., and Kervina, M. (1974) *Methods Enzymol.* **31,** 6–41
Gadaleta, G., Pepe, G., De Candia, G., Quagliariello, C., Sbisà, E., and Saccone, C. (1989) *J. Mol. Evol.* **28,** 497–516
Goglia, F., Torresani, J., Bugli, P., Barletta, A., and Liverini, G. (1981) *Pfluegers Arch. Eur. J. Physiol.* **390,** 120–124
Goldberg, Y., Glineur, C., Gesquiere, J. C., Riconart, A., Sap, J., Vennström, B., and Ghysdael, J. (1988) *EMBO J.* **7,** 2425–2433
Graupner, G., Wills, K. N., Tzukerman, M., Zhang, X., and Pfahl, M. (1989) *Nature* **340,** 653–656
Greenawalt, J. W. (1974) *Methods Enzymol.* **31,** 310–323
Gustafsson, R., Tata, J. R., Lindberg, J., and Ernster, L. (1965) *J. Cell Biol.* **26,** 555–578
Hafner, P. R., Brown, G. C., and Brand, M. D. (1990) *Biochem. J.* **265,** 731–734
Hashizume, K., and Ichikawa, K. (1982) *Biochem. Biophys. Res. Commun.* **106,** 920–926
Höger, T. I., Grund, C., Franke, W. W., and Krohne, G. (1991) *Europ. J. Cell Biol.* **54,** 150–156
Horowitz, Z. D., and Samuels, H. H. (1988) *Affinity Labeling and Cloning of Steroid and Thyroid Hormone Receptors* (Gronemeyer, H., ed) pp 79–83, Ellis Horwood Ltd, Chichester, UK
Jakovilcic, S., Swift, H. H., Gross, N. J., and Rabinowitz, R. (1978) *J. Cell Biol.* **77,** 887–901
Johnson, L. V., Walsh, M. L., and Chen, L. B. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 990–994
Kadenbach, B. (1966) *Regulation of Metabolic Processes in Mitochondria* (Targer, J. M., Papa, S., Quagliariello, E., and Slater, E. C., eds) pp 508–517, Elsevier Science Publishers B.V., Amsterdam
Laemmli, U. K. (1970) *Nature* **227,** 680–685
Lin, K. H., Ashizawa, K., and Cheng, S. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 7737–7741
Martino, G., Covello, C., De Giovanni, R., Filipelli, R., and Pitrelli, G. (1986) *Mol. Biol. Rep.* **11,** 205–211
Morré, D. J. (1971) *Methods Enzymol.* **22,** 130–145
Mutvei, A., Husman, B., Andersson, G., and Nelson, B. D. (1989a) *Acta Endocrinol.* **121,** 223–228
Mutvei, A., Kuzela, S., and Nelson, B. D. (1989b) *Eur. J. Biochem.* **180,** 235–240
Ragan, C. I., Wilson, M. T., Darley-Usmar, V. M., and Lowe, P. M. (1987) *Mitochondria: A Practical Approach* (Darley-Usmar, V. M., Rickwood, D., and Wilson, M. T., eds) pp 79–112, IRL Press, Oxford
Rasmussen, U. B., Köhrle, J., Rokos, H., and Hesch, R. D. (1989) *FEBS Lett.* **255,** 385–390
Rodd, C., Schwartz, H. L., Strait, K. A., and Oppenheimer, J. H. (1992) *Endocrinology* **131,** 2559–2564
Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Coning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Sap, J., Munoz, A., Damm, K., Goldberg, Y., Ghysdael, J., Leutz, A., Beug, H., and Vennström, B. (1986) *Nature* **324,** 635–640
Schwartz, H. L., Strait, K. A., Ling, N. C., and Oppenheimer, J. H. (1992) *J. Biol. Chem.* **267,** 11794–11799
Soboll, S., Horst, C., Hummerich, H., Schumacher, J. P., and Seitz, H. J. (1992) *Biochem. J.* **281,** 171–173
Sterling, K. (1986) *Endocrinology* **119,** 292–295
Sterling, K., and Milch, P. O. (1975) *Proc. Natl. Acad. Sci U. S. A.* **72,** 3225–3229
Sterling, K., Milch, P. O., Brenner, M. A., and Lazarus, J. H. (1977) *Science* **197,** 996–999
Sterling, K., Lazarus, J. H., Milch, P. O., Sakurada, T., and Brenner, M. A. (1978) *Science* **201,** 1126–1129
Sterling, K., Brenner, M. A., and Sakurada, T. (1980) *Science* **210,** 340–343
Sterling, K., Campbell, G. A., and Brenner, M. A. (1984a) *Acta Endocrinol.* **105,** 391–397
Sterling, K., Campbell, G. A., Taliadouros, G. S., and Nunez, E. A. (1984b) *Cell Tissue Res.* **236,** 321–325
Szczesna-Kaczmarek, A. (1990) *Intl. J. Biochem.* **22,** 617–620
Van Itallie, C. M. (1990) *Endocrinology* **127,** 55–62
Wharton, D. C., and Tzagaloff, A. (1967) *Methods Enzymol.* **10,** 245–250