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The Site of Synthesis of the Iron-Sulfur Subunits of the Flavoprotein and Iron-Protein Fractions of Human NADH Dehydrogenase*

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The site of synthesis of the iron-sulfur subunits of the flavoprotein and iron-protein fractions of the human respiratory chain NADH dehydrogenase has been investigated to test the possibility that any of them is synthesized in mitochondria. For this purpose, antibodies specific for individual subunits of the bovine enzyme, which cross-reacted with the homologous human subunits in immunoblot assays, were tested against HeLa cell mitochondrial proteins labeled *in vivo* with [^{35}S]methionine in the absence or presence of inhibitors of mitochondrial or cytoplasmic protein synthesis. The results clearly indicated that all the iron-sulfur subunits of the flavoprotein and iron-protein fractions of human complex I are synthesized in the cytosol and are, therefore, encoded in nuclear genes.

The NADH-ubiquinone oxidoreductase is the most complicated among the enzymes of the mitochondrial respiratory chain (1). It has been isolated from bovine heart as a large structure (complex I, Ref. 2), consisting of about 25 polypeptides (3) and phospholipids (4), and capable of reducing ubiquinone analogues by the physiological, rotenone-sensitive pathway (1, 5). The analysis of the structure and function of the beef heart enzyme has been greatly facilitated by the use of chaotropic agents (6), which can resolve complex I into three fractions: *i.e.* the low molecular weight NADH dehydrogenase or flavoprotein (FP)[1] fraction, which retains NADH dehydrogenase activity with a variety of artificial electron acceptors and contains the FMN and two iron-sulfur centers (7–10), the iron-protein (IP) fraction, which contains four iron-sulfur centers (10, 11), and the hydrophobic protein (HP) fraction, which contains two or three iron-sulfur centers (9). The FP consists of three polypeptides (51, 24, and 9 kDa) (3, 7); the IP fraction consists of six to eight polypeptides, including those associated with the iron-sulfur centers, of molecular masses of 75, 49, 30, and 13 kDa, plus several minor components (3, 10, 12); and the HP fraction consists of at least 15 hydrophobic polypeptides and of phospholipids (3, 5).

Recently, by the use of antibodies against highly purified native beef heart complex I or against the isolated 49-kDa iron-sulfur protein and by enzyme fractionation studies, it has been shown that seven subunits of the human respiratory chain NADH dehydrogenase are encoded in mtDNA, being specified by the unidentified reading frames URF1, URF2, URF3, URF4, URF4L, URF5, and URF6 (13–15). (These reading frames have therefore been renamed ND1 (for NADH dehydrogenase 1), ND2, ND3, ND4, ND4L, ND5, and ND6). Support for this conclusion has subsequently come from the finding that the purified rotenone-sensitive NADH dehydrogenase from *Neurospora crassa* contains several subunits synthesized within the mitochondria (16) and from the observation that the stopper mutant of *N. crassa*, whose mtDNA lacks two genes homologous to ND2 and ND3, has no functional complex I (17, 18).

The above-mentioned work did not provide any evidence as to the nature of the mtDNA-encoded components of complex I. More recently, however, by immunoblot assays using SDS-denatured fractions of beef heart complex I and antibodies raised against peptides of the human ND gene products which are well conserved in the homologous bovine subunits, it has been shown that three of these products, *i.e.* those of ND1, ND3, and ND4L, are components of the HP fraction (19, 20). Among the other ND gene products, one, *i.e.* that of ND5, contains 4 cysteine residues conserved in all vertebrate mtDNAs so far sequenced (21–24)[2]; these cysteines form two pairs, being separated in each pair by 11 amino acids, and the two pairs are 37–38 amino acids apart in different species. This remarkably conserved regular arrangement of cysteines suggests that the ND5 gene product, or a derivative thereof, may be an iron-sulfur subunit of complex I. Another ND gene product, that of ND4, also contains 2 cysteine residues separated by 51 amino acids, which are conserved in all vertebrate and invertebrate mtDNAs so far analyzed. It is interesting that if the ND4 gene product formed a dimer, the two molecules could conceivably provide the cysteine ligands for an iron-sulfur cluster binding site, with an overall arrangement of cysteines very similar to that of the putative cluster binding site of the ND5 product. Furthermore, the possibility has to be considered that two non-cysteine ligands of the ND4 gene product may be used with the two cysteines for the coordination of a binuclear iron-sulfur cluster, as reported for the Rieske iron-sulfur protein isolated from *Thermus thermophilus* (25).

Some of the conserved cysteine residues of the ND4 and ND5 genes which were mentioned above are not found in mtDNA from *Drosophila* species (26) or from lower eukaryotes, like *Aspergillus nidulans* (27), *Trypanosoma brucei* (28), and *Chlamydomonas reinhardtii* (29). However, the ND genes are the most rapidly diverging among the mitochondrial genes, and the amino acid sequence similarity of the ND4 and ND5 genes from the species cited above with the homologous mammalian genes is very low (20–32.5%). If the ND4 and ND5 gene products are iron-sulfur proteins, the absence of some of the cysteines in the products from the above cited organisms may reflect a late acquisition in the course of

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[1] The abbreviations used are: FP, flavoprotein; IP, iron-protein fraction; HP, hydrophobic protein; SDS, sodium dodecyl sulfate.

[2] G. Gadaleta and C. Saccone, personal communication.

evolution of the redox functions of these ND gene products. In fact, the NADH dehydrogenase may be structurally different in the above cited lower eukaryotes as has been well documented in *S. cerevisiae*. Alternatively, it is possible that some of the cysteines can be substituted by non-cysteine ligands as in the example discussed above.

Among the iron-sulfur subunits of complex I, the best characterized from the functional point of view are the 51-, and 24-kDa subunits of the FP fraction (3, 7) and the 75-, 49-, 30-, and 13-kDa subunits of the IP fraction (3, 10, 12). While the reported amino acid composition of the 51-kDa subunit (7) and the amino acid sequence of the 24-kDa subunit (30) suggest strongly that these subunits are encoded in nuclear genes, no information is available on the genetic origin and site of synthesis of the four iron-sulfur subunits of the IP fraction. In the present work, the possibility that the human ND5 and/or ND4 gene product or derivatives thereof correspond(s) to iron-sulfur subunits of this fraction of NADH dehydrogenase has been investigated by examining the site of synthesis of the latter polypeptides in HeLa cells. At the same time, it was thought desirable to verify experimentally the cytosolic site of synthesis of the 51- and 24-kDa subunits which was suggested by structural data. It has been found that all iron-sulfur subunits of the FP and IP fractions of human complex I are synthesized in the cytosol and are therefore encoded in nuclear genes.

MATERIALS AND METHODS

Preparation of Antisera—Reference is made to previous work for the purification of bovine complex I (3) and for the isolation from it of the FP fraction (6). The isolation of the 51- and 24-kDa subunits from the FP fraction of the complex and that of the 75-, 49-, 30-, and 13-kDa subunits from the IP fraction, and the preparation of subunit-specific antisera were carried out as detailed elsewhere (8, 10, 20, 31). The antisera against the 51- and 24-kDa subunits were prepared using the subunits purified by electrophoresis and electroelution from the FP fraction (32); for the affinity purification of the anti-51-kDa antibodies, see below.

Labeling Conditions—Exponentially growing HeLa cells were pelleted, resuspended in methionine-free medium supplemented with 5% dialyzed calf serum at 2–3 × 10^6 cells/ml, and labeled with [^{35}S] methionine (9–125 μCi/ml; 1200–1500 Ci/mmol) for 2.5 h either in the absence of inhibitors, or in the presence of the mitochondrial protein synthesis inhibitor chloramphenicol at 100 μg/ml or of a cytoplasmic protein synthesis inhibitor, emetine or cycloheximide, at 100 μg/ml. (The cycloheximide pulse was followed by an 18-h chase in the absence of the drug in methionine-containing medium (15).)

Immunoprecipitation Experiments—Samples of an SDS mitochondrial lysate from cells labeled according to one of the protocols described above were incubated with each of the subunit-specific antisera or with normal serum, and the immunoprecipitates were isolated according to a previously described procedure (33). The immunoprecipitated products were run on SDS, 8 M urea, 15% polyacrylamide gels (34), unless otherwise specified.

Immunoblot Assays—Samples of beef heart or human heart submitochondrial particles, prepared as previously described (12), of beef heart complex I, or of HeLa cell mitochondria were dissociated in 1% SDS, 1% β-mercaptoethanol, 0.005 M *p*-aminobenzamidine (to inhibit proteases), and run on a 12–16% SDS-polyacrylamide gel in the Laemmli buffer system (35). The proteins were electrophoretically transferred to nitrocellulose (36) and treated with a mixture of monospecific antisera to the 75-, 49-, 30-, and 13-kDa subunits of the IP fraction of beef heart complex I (31), with an anti-49-kDa antiserum, or with an antiserum against the FP fraction, as detailed in the figure legends. Antibody binding was visualized with ^{125}I-labeled protein A and autoradiography (31), with biotinylated donkey anti-rabbit Ig and streptavidin-biotin-peroxidase complex (37), or with goat anti-rabbit Ig-alkaline phosphatase complex (38), as specified below.

Affinity Purification of Antibodies—For affinity purification of anti-51-kDa antibodies from an antiserum prepared against the native FP fraction, the method of Bisson and Schiavo (39) was used, with some modifications. In particular, after electrophoresis of a sample of purified FP fraction in an SDS, 10% polyacrylamide gel in the Laemmli buffer system, the subunits of this fraction were transferred onto a nitrocellulose sheet and stained with a 0.1% Amido Black solution in 45% (v/v) methanol, 10% (v/v) acetic acid, for 2 min. After destaining, a strip containing the 51-kDa protein was cut out, washed in distilled water, and then incubated overnight with phosphate-buffered saline containing 0.5% bovine serum albumin, and subsequently for 1 h with 2 ml of a 1:20 dilution in phosphate-buffered saline of an antiserum against the FP fraction. The strip was washed with the 0.5% bovine serum albumin solution in phosphate-buffered saline, and the bound antibodies were then eluted by incubating the strip in 1 ml of 0.2 M HCl-glycine, pH 2.2, containing 3% bovine serum albumin, at 4 °C for 4 min. The solution, after removal of the strip, was rapidly brought to pH 7.5 with 1 M Tris base.

RESULTS AND DISCUSSION

Immunological Cross-reactivity of Iron-Sulfur Proteins from Beef Heart and Human Complex—In previous work, antisera specific for the 75-, 49-, 30-, or 13-kDa subunit of the IP fraction of bovine complex I were raised in rabbits by using as antigens the individual subunits purified by preparative gel electrophoresis (31). Immunoblot assays showed that each antiserum identified only a single polypeptide of the appropriate molecular mass; the only exception was the anti-75-kDa antiserum, which, in some preparations, also reacted with minor components of smaller molecular mass than 75 kDa, which were probably proteolytic breakdown products of this protein (31). In further work, each subunit-specific antiserum was shown to cross-react with a single protein from submitochondrial particles of different mammalian species, including human, which had an identical or very similar molecular mass to that of the bovine iron-sulfur protein used as an antigen, and which represented presumably the homologous subunit (40). Fig. 1*a* shows the results of an immunoblot experiment in which the proteins from beef heart (*B*) or human heart (*H*) submitochondrial particles, electrophoretically fractionated in an SDS-polyacrylamide gel and transferred onto nitrocellulose, were tested in parallel for their reactivity with a mixture of the anti-75-, anti-49-, anti-30-, and anti-13-kDa antisera. One can see that, in the human sample, three polypeptides with the same electrophoretic mobilities as those of the 75-, 49-, and 13-kDa bovine iron-sulfur proteins and one polypeptide moving somewhat faster than the bovine 30-kDa subunit (estimated mass ~28 kDa (33)) reacted with the antisera. The cross-reactivity appears to be particularly strong for the 49- and ~30-kDa human proteins. These results indicated that the bovine subunit-specific antisera recognized the homologous proteins from human submitochondrial particles and could therefore be utilized to test the site of synthesis of the human iron-sulfur proteins.

Monospecific antisera raised against the purified 51- or 24-kDa iron-sulfur protein of the flavoprotein fraction of bovine complex I (20) reacted positively, but only weakly (especially the anti-51-kDa antiserum), with proteins of the same molecular mass from HeLa cell mitochondria. As an alternative to the anti-51-kDa antiserum, an antiserum against the bovine FP fraction, which by immunoblot assays had been shown to contain 51-kDa subunit-specific antibodies cross-reacting with the putative homologous protein from HeLa cell mitochondria, was used to test the site of synthesis of the 51-kDa subunit. As shown in Fig. 1*b*, the beef heart complex I sample (*B*), when tested with this antiserum, exhibited a strong and a relatively weak band, which, in other runs, were found to correspond in migration to the 51-kDa component, and, respectively, the 24-kDa component of bovine complex I run in a parallel lane (not shown). The sample of HeLa cell mitochondrial proteins (*H*) exhibited clearly a band corresponding

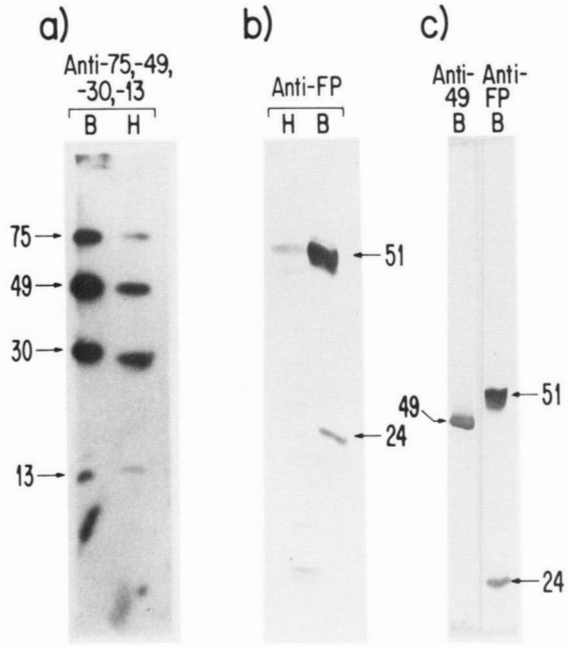


FIG. 1. **Immunological cross-reactivity of iron-sulfur proteins of bovine and human NADH dehydrogenase.** *a*, immunoblots of the proteins of submitochondrial particles from beef heart (*B*) and human heart (*H*) assayed with a mixture of rabbit monospecific antisera against the 75-, 49-, 30-, and 13-kDa iron-sulfur subunits from the IP fraction of bovine NADH dehydrogenase. Samples of submitochondrial particles (12) were dissociated in 1% SDS, 1% mercaptoethanol, 5 mM *p*-aminobenzamidine (to inhibit proteases). 50-μg portions were run on a 12–16% polyacrylamide gradient gel, and the proteins were transferred electrophoretically onto a nitrocellulose membrane, and then tested with a mixture of appropriate dilutions of the antisera; antibody binding was visualized with ^{125}I-labeled protein A and autoradiography. *b*, immunoblots of beef heart complex I (*B*) and human mitochondria (*H*) assayed with an antiserum against beef heart FP fraction. A 35-μg sample of complex I and a 200-μg sample of HeLa cell mitochondria were dissociated as described in *a*, run in a 10% polyacrylamide gel, transferred electrophoretically onto a nitrocellulose membrane and tested with a 1:100 dilution of the antiserum; antibody detection was by alkaline phosphatase-conjugated second antibody. *c*, immunoblots of beef heart complex I assayed with an anti-49-kDa antiserum or an anti-FP antiserum. 35-μg samples of complex I were electrophoresed, electroblotted, and tested with the antisera as described in *b*; antibody detection was by the streptavidin-biotin-peroxidase system.

in migration to the bovine 51-kDa subunit; in addition, several minor faster moving bands, of uncertain significance, could be seen. In another immunoblot experiment, in which two samples of beef heart complex I, electrophoresed in parallel, were tested with the anti-49-kDa antiserum or with the antiserum against the FP fraction, it was shown that the latter antiserum lacked antibodies directed against the 49-kDa subunit, which was well separated under the electrophoretic conditions used (Fig. 1*c*).

Site of Synthesis of the Iron-Sulfur Subunits of the IP Fraction—Figs. 2 and 3 show the electrophoretic patterns in SDS-urea-polyacrylamide gels of immunoprecipitates obtained by incubating samples of SDS mitochondrial lysates from HeLa cells, labeled with [^{35}S]methionine for 2.5 h in the absence or presence of protein synthesis inhibitors, with monospecific antisera directed against the beef heart 49-, 30- (Fig. 2), 13-, and 75-kDa iron-sulfur proteins (Fig. 3). The precipitate produced by the anti-49-kDa antiserum incubated with the mitochondrial lysate from cells labeled in the absence of inhibitors exhibits two closely migrating bands, each band consisting of a doublet of sharp bands (Fig. 2*a*). From their mobility relative to that of water-soluble markers, the estimated molecular mass of these components can be estimated to be ~50 kDa. The same components can be seen in the precipitate produced by the anti-49-kDa antiserum incubated with a mitochondrial lysate from cells labeled in the presence of chloramphenicol at 100 μg/ml. No such labeled components can, on the contrary, be observed in the precipitate obtained from the lysate of cells labeled in the presence of cycloheximide at 100 μg/ml. These results clearly indicated that the ~50 kDa components are synthesized in the cytosol. It seemed likely that the multiple bands precipitated by the anti-49-kDa antiserum corresponded to different forms of the same protein resulting from secondary modifications. Indeed, on reduction and alkylation of the cysteine residues, the immunoprecipitated protein was found to migrate as a single band in an SDS-urea-polyacrylamide gel, indicating that the multiplicity of bands was probably due to intramolecular disulfide bond formation (Fig. 2*a*). When the protein precipitated by the anti-49-kDa antiserum was run in an SDS-polyacrylamide gel, only one component of the expected mobility was observed (data not shown).

Similar experiments to those described above were carried out with the anti-30-kDa and the anti-13-kDa antisera. These experiments indicated the presence in the mitochondrial lysate from HeLa cells of a protein of approximately 25 kDa molecular mass (Fig. 2*b*) and, respectively, of a component of ~13 kDa molecular mass (Fig. 3*a*), as estimated from their mobilities relative to those of water-soluble markers. These proteins were labeled in the absence of inhibitors or in the presence of chloramphenicol, but not in the presence of cycloheximide, clearly pointing to a cytoplasmic site of synthesis.

The precipitate produced by the anti-75-kDa antiserum incubated with the mitochondrial lysate from cells labeled in the absence of inhibitors or in the presence of chloramphenicol exhibited a major band, appearing as a doublet, which, by comparison with the migration of water-soluble markers could be estimated to have a molecular mass of ~75 kDa (Fig. 3*b*). A minor band, corresponding to a component of ~86 kDa, and some heterogeneous material migrating between the ~75 kDa and the ~86 kDa bands or slower than the 86 kDa band, could also be seen. The ~75 kDa and the ~86 kDa bands, as well as the heterogeneous material were absent in the precipitate obtained with a lysate from cells labeled in the presence of cycloheximide. These results clearly indicated that both the ~75- and the ~86-kDa components, as well as the heterogeneous material are synthesized in the cytosol. After reduction and alkylation of the immunoprecipitate before electrophoresis, both the ~75 kDa and the ~86 kDa bands persisted, and they were indeed more prominent due to the disappearance of the heterogeneous material in the background; furthermore, the ~75-kDa doublet now appeared as a single band (Fig. 3*c*). These results suggested that both the heterogeneous material and the doublet appearance of the ~75 kDa band were due to aggregation and intramolecular disulfide bond formation. The ~75-kDa component presumably corresponds to the bovine 75-kDa iron-sulfur protein. The nature of the 86-kDa component is, on the contrary, uncertain. In an SDS-polyacrylamide gel, the immunoprecipitate exhibited only one component (Fig. 3*d*). It seems possible that the 86-kDa component results from aggregation of the 75-kDa protein with some other component in the presence of urea. It is known that urea reduces the affinity of proteins for SDS (42), and may thus facilitate their aggregation with other proteins.

In the experiments of Figs. 2 and 3, no other components besides those described above were observed in the electrophoretic pattern of the immunoprecipitate, even after long

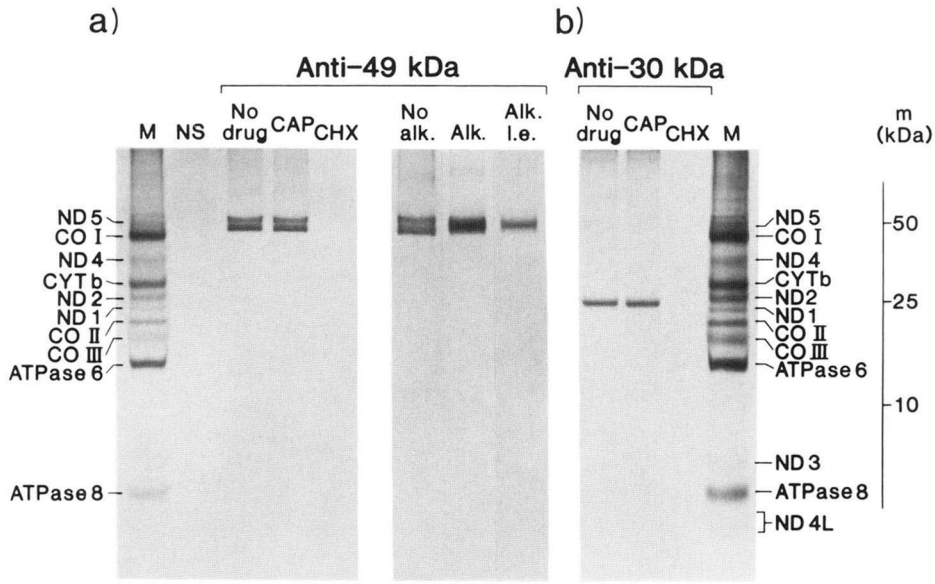
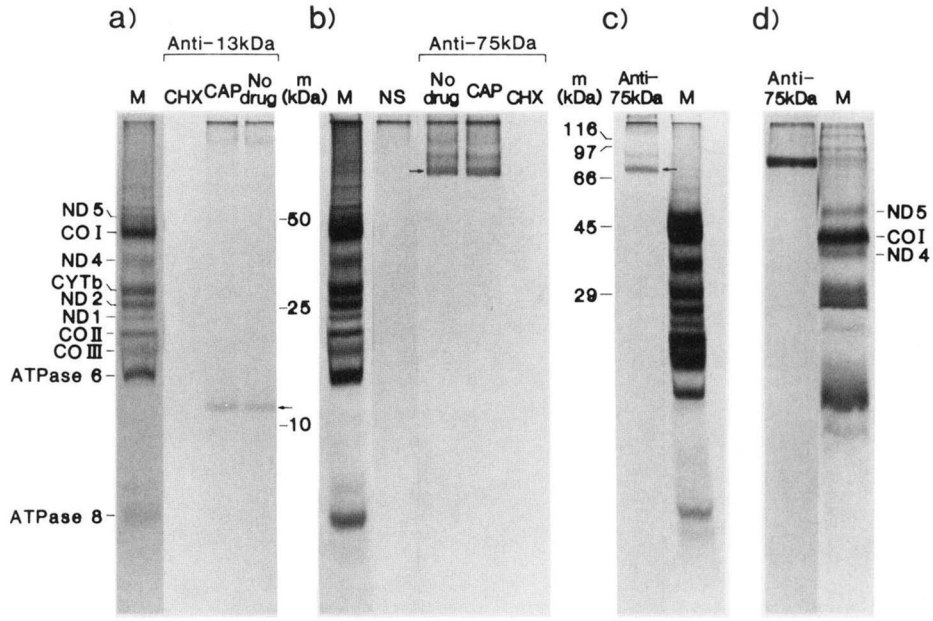


FIG. 2. **Sensitivity to inhibitors of protein synthesis of the labeling with [^{35}S]methionine of the 49-kDa (*a*) and the 30-kDa iron-sulfur subunit (*b*) in HeLa cells.** Samples of an SDS mitochondrial lysate from Hela cells labeled for 2.5 h with [^{35}S]methionine in the absence of drug (*No drug*) or in the presence of 100 μg/ml chloramphenicol (*CAP*) or 100 μg/ml cycloheximide (*CHX*) were incubated with an anti-49-kDa antiserum or an anti-30-kDa antiserum, or with normal serum (*NS*), and the immunoprecipitates were run in SDS-urea-polyacrylamide gels. In the right panel of *a*, samples of the precipitate obtained with anti-49-kDa antibodies from the mitochondrial lysate of cells labeled in the absence of inhibitors were run on the gel without any treatment (*No alk.*) or after reduction and alkylation with iodoacetamide (*Alk.*) (*l.e.*, low exposure of the alkylated sample). *M*, pattern of HeLa cell mitochondrial translation products; *CO I, CO II, CO III*, subunits I, II, and III of cytochrome *c* oxidase; *CYTb*, apocytochrome *b*; *ATPase 6* and *ATPase 8*, subunits 6 and 8 of the H^+-ATPase; *ND1, ND2, ND3, ND4, ND4L, ND5*, and *ND6*, subunits of NADH dehydrogenase. The molecular mass (*m*) scale on the right was based on the mobilities of the mitochondrial translation products, previously calibrated against water-soluble molecular weight markers (33, 41).

FIG. 3. **Sensitivity to inhibitors of protein synthesis of the labeling with [^{35}S]methionine of the 13-kDa (*a*) and the 75-kDa iron-sulfur subunit (*b, c,* and *d*) in HeLa cells.** Samples of an SDS mitochondrial lysate from Hela cells labeled as in the experiments illustrated in Fig. 2 were incubated with an anti-13-kDa antiserum or an anti-75-kDa antiserum, or with normal serum (*NS*). The immunoprecipitates were run in SDS-urea-polyacrylamide gels in *a, b,* and *c*, in an SDS-polyacrylamide gradient gel in *d*. In *c*, the immunoprecipitate was run on gel after reduction and alkylation with iodoacetamide. Explanations of symbols as in Fig. 2. The molecular mass scale in *panel a* was derived as explained in the legend of Fig. 2; the molecular masses in *panel c* pertain to protein markers (β-galactosidase (116,000), phosphorylase b (97,000), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (29,000)), run in a separate lane and stained with Coomassie Blue.



exposure of the autoradiograms. On account of this specificity and because of the correspondence in size of the immunoprecipitated polypeptides to the components detected in the immunoblot experiment (Fig. 1), it is reasonable to conclude that the polypeptides specifically reacting with the antibodies raised against the 75-, 49-, 30-, or 13-kDa bovine iron-sulfur protein represent the homologous human proteins. To be noticed is that, in both the immunoblot and in the immunoprecipitation experiments, the human subunit corresponding to the bovine 30-kDa iron-sulfur protein migrated as a somewhat smaller protein. In the gel system used here, the estimated mass was ≃25 kDa; previously, in a different gel system, a mass of 28 kDa had been estimated for the human subunit (40).

Site of Synthesis of the Iron-Sulfur Subunits of the FP Fraction—As mentioned above, the antisera prepared against the bovine 51- and 24-kDa iron-sulfur proteins of the flavoprotein fragment of bovine complex I exhibited only a weak

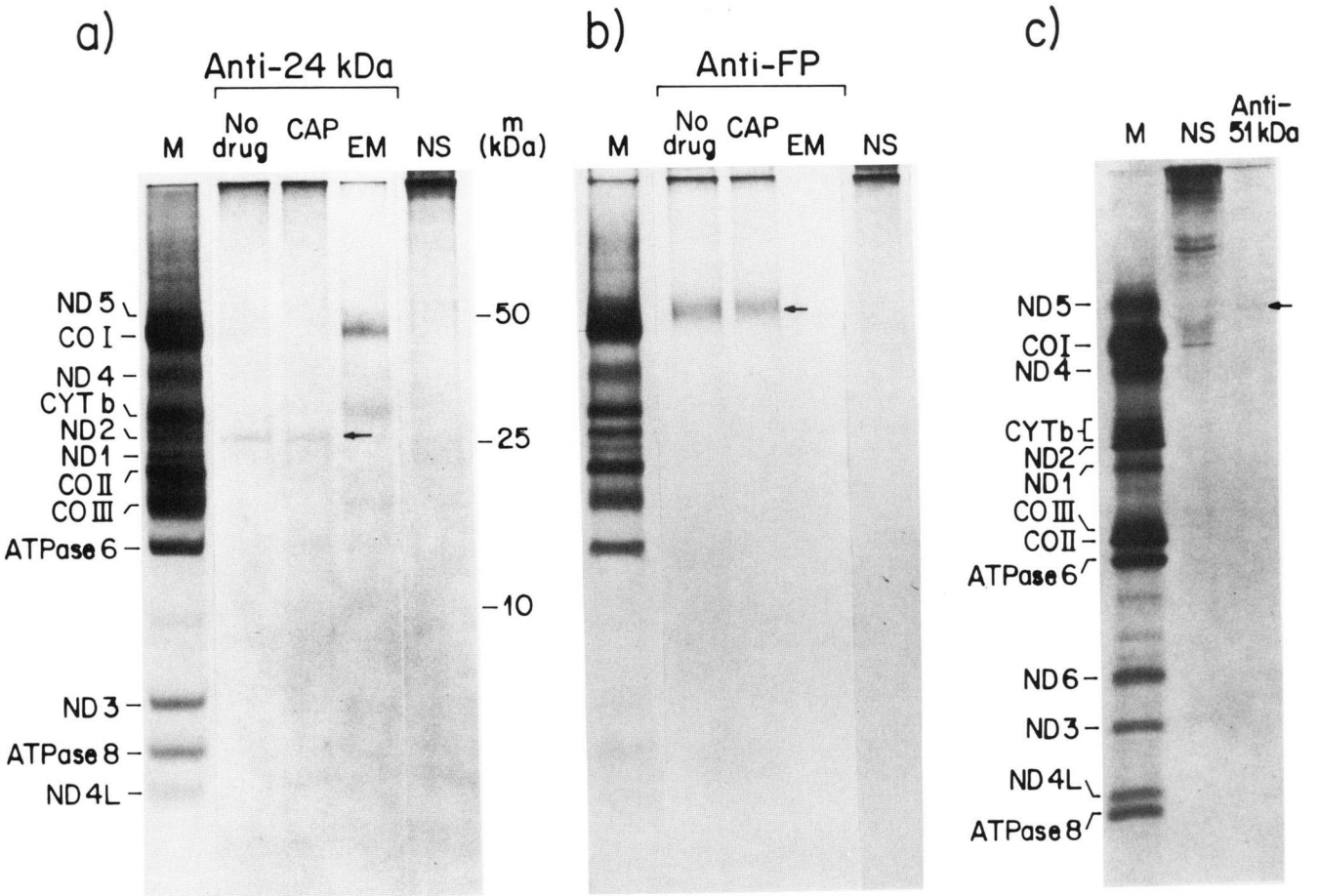


FIG. 4. **Sensitivity to inhibitors of protein synthesis of the labeling with [^{35}S]methionine of the 24-kDa (*a*) and the 51-kDa iron-sulfur subunits (*b* and *c*) in HeLa cells.** Samples of an SDS mitochondrial lysate from HeLa cells labeled as in the experiments of Fig. 2 were incubated with an anti-24-kDa antiserum (*a*), or with an anti-FP antiserum (*b*), or with affinity-purified anti-51-kDa antibodies (*c*), or with normal serum (*NS*). *EM,* emetine; explanation of other symbols as in Fig. 2.

cross-reactivity with the homologous human subunits in immunoblot assays. Nonetheless, they were tested in immunoprecipitation experiments with mitochondrial lysates from [^{35}S]methionine-labeled HeLa cells. Indeed, as shown in Fig. 4*a*, the precipitate produced by the anti-24-kDa antiserum, incubated with a mitochondrial lysate from cells labeled in the absence of inhibitors or in the presence of chloramphenicol, exhibited a single weakly labeled component with an estimated molecular mass of ~25 kDa, which was absent in the precipitate obtained with a lysate from cells labeled in the presence of emetine. Due to the long exposure time of the autoradiogram, the latter precipitate showed bands representing a background of selectively labeled mitochondrial translation products adsorbed on the *Staphylococcus aureus* used to isolate the antigen-antibody complexes. The absence of these bands in the sample incubated in the absence of inhibitors (no drug) is due to the much lower specific activity of the mitochondrial translation products under these conditions.[3] Although the component precipitated by the anti-24-kDa antibodies gave only a weak signal, its specificity and its correspondence in size to the bovine 24-kDa iron-sulfur protein of the FP fraction strongly suggests that it represents the homologous human protein.

The precipitate produced by anti-51-kDa antiserum incubated with the mitochondrial lysate from cells labeled in the absence of inhibitors or in the presence of chloramphenicol exhibited a single very weakly labeled band corresponding to a component with an estimated molecular mass of ~50 kDa, which was absent in the precipitate obtained with a lysate labeled in the presence of emetine (not shown). The anti-51-kDa antibodies present in the anti-FP antiserum turned out, on the contrary, to be much more effective in precipitating the putative 51-kDa subunit, whose labeling was chloramphenicol-resistant and emetine-sensitive (Fig. 4*b*). In support of this identification, 51 kDa-specific antibodies isolated from the anti-FP antiserum by affinity purification, when tested with the mitochondrial lysate from cells labeled in the presence of chloramphenicol, precipitated the same component (Fig. 4*c*).

Conclusions—The evidence presented above indicates that the six iron-sulfur subunits of the FP and IP fractions of human complex I are all synthesized in the cytosol and that, therefore, none of them can be a product of a mitochondrial gene, in particular ND5 or ND4. The above conclusion, as concerns the 51- and 24-kDa iron-sulfur subunits of the FP fraction, was implicit in the previously reported amino acid composition (7) and, respectively, sequence of the protein (30). Recently, precursor forms of the 75- and 49-kDa subunits have been identified with specific antibodies in a bovine kidney cell line labeled in the presence of an uncoupler to inhibit mitochondrial import[4]; this result confirms the nuclear origin of these two iron-sulfur proteins.

[3] A. Chomyn, unpublished observations.

[4] G. Gibb and C. I. Ragan, unpublished observations.

It is interesting that, in the rotenone-sensitive NADH dehydrogenase, most, if not all, of the major catalytic subunits are encoded in the nuclear genome and synthesized in the cytosol, in contrast to the situation in cytochrome *c* oxidase. The information which is emerging from the present and previous work (14, 15) concerning the respective role of the cytosolic and the mitochondrial compartment in the synthesis of subunits of the NADH dehydrogenase has relevance to understanding the mode of assembly of this large complex. Furthermore, this information has to be seen in the context of what is known about the arrangement in the complex of the subunits synthesized in the cytosol and those synthesized within the organelle. A variety of approaches, including fragmentation with chaotropic agents and chemical modification of isolated complex I or intact membranes with hydrophilic and hydrophobic probes have led to a model of complex I in which the polypeptides of the HP fraction, which include most if not all the mitochondrially synthesized subunits (14, 15, 20), form a shell interacting with the lipid bilayer and surrounding the hydrophilic FP and IP fractions (20). On the other hand, labeling studies have shown that the 75-, 49-, and 30-kDa iron-sulfur subunits of the IP fraction are in part accessible to hydrophilic probes in the intact membrane, as well as in the isolated enzyme, and therefore must protrude on one or both sides of it (9, 20). In agreement with this conclusion, it has been shown that antibodies against the purified 49-kDa iron-sulfur protein can precipitate from a Triton X-100 lysate of HeLa cell mitochondria a complex containing all the ND gene products (15).

According to the model outlined above, the main function of the subunits of the HP fraction would be to form a hydrophilic pocket within the inner membrane to accommodate the two hydrophilic domains of the enzyme. However, some of the hydrophobic polypeptides of the shell must also participate in the catalytic function of the NADH dehydrogenase. In particular, the redox centers of the HP fraction are probably involved in electron and proton transfer to ubiquinone in the hydrophobic environment of the membrane. In agreement with this idea is the recent observation that one of the mtDNA-encoded polypeptides of the HP fraction (the ND1 gene product) is the rotenone-binding protein (43). Since the rotenone-binding site is close to that of ubiquinone, the ND1 gene product may well be involved in ubiquinone reduction. If the ND5 and/or ND4 gene products are indeed iron-sulfur proteins, they may also participate in this process. Furthermore, a potential NAD/NADH-binding domain has been identified within the human ND6 product, based on certain structural features it shares with known NAD/NADH-binding proteins (44).

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