Identification of the subunits of bovine NADH dehydrogenase which are encoded by the mitochondrial genome

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Products of the mitochondrial genome were identified in the bovine kidney cell line NBL-1 by labelling with [³⁵S]methionine in the presence of cycloheximide. Seven proteins were precipitated by an antiserum to bovine heart NADH dehydrogenase, corresponding to the seven mitochondrial gene products identified in the human HeLa cell line. Comparison of these mitochondrial gene products with purified bovine NADH dehydrogenase by SDS/gel electrophoresis revealed that the ND-5 product is probably a previously unidentified protein of apparent M_r 51 000, and the ND-4 product is the protein of apparent M_r 39 000.

INTRODUCTION

In recent years, a great deal of attention has been directed towards identifying all of the information encoded within the mammalian mitochondrial genome [1–4]. Although mitochondrial DNA of higher eukaryotes has the potential to encode thirteen different polypeptides, until 1986 only six had been positively identified [1–5]. The remaining unidentified reading frames (URFs) code for products that are highly hydrophobic [6]. A strong candidate to contain some or all of the URFs was NADH dehydrogenase (Complex I) which consists of a shell of hydrophobic proteins surrounding a core containing the flavoprotein and iron–protein fragments [7, 8]. Attardi *et al.* [9] confirmed the identity of a further six URFs in human cells by utilizing antibodies against purified bovine heart Complex I and against synthetic peptides predicted from the human URF sequences. These polypeptides were subsequently renamed ND (for NADH dehydrogenase) 1, 2, 3, 4, 4L and 5. The last URF (URF 6) has more recently been identified by use of antibodies directed against peptides predicted from the DNA sequence [10]. In the same study, it was shown that URF 6 was a component of NADH dehydrogenase from immunoprecipitation studies with antisera against native bovine heart Complex I and the M_r -49 000 nuclear-encoded component of the complex. Its previous identification as a component of NADH dehydrogenase was hindered by its sensitivity to the source of SDS used in the electrophoretic analysis.

On SDS/gel electrophoresis, especially in the presence of urea [9], the ND gene products migrate with apparent M_r values which are lower than the real values [9,10]. However, the seven human ND products behave similarly in that the logarithms of the electrophoretic mobilities are linearly related to the true M_r values, even in gel systems where the apparent M_r values vary considerably [9]. This variability makes it impossible to identify the ND gene products with particular polypeptides of the purified enzyme on the basis of apparent M_r values obtained in different laboratories. Moreover, the only purified preparation of the enzyme is from bovine heart,

and the bovine ND gene product sequences, although very similar, are not identical. Thus the apparent M_r values of the bovine proteins are unlikely to be exactly the same as for their human counterparts, and, given the large number of subunits of mammalian NADH dehydrogenase, a side-by-side comparison of the ND gene products and purified enzyme from the same species is essential for clear identification.

In this paper, we have used a bovine cell line (NBL-1) to study the ND gene products. We were able to identify the same seven ND products that were found in the HeLa cell line and, by co-electrophoresis with purified NADH dehydrogenase, we have identified the subunits of the enzyme which are mitochondrially encoded.

MATERIALS AND METHODS

Materials

The NBL-1 kidney cell line and Ham's F-12 medium were supplied by Flow Laboratories. A 10% (w/v) suspension of *Staphylococcus aureus* cells, Cowan I strain (Protein A, bacterial adsorbant) and Trans-³⁵S-label (> 1000 Ci/mmol) were purchased from ICN Bio-medicals Inc. X-ray film (Hyperfilm-MP) was obtained from Amersham International. Cycloheximide, chloramphenicol and digitonin were obtained from Sigma Chemical Co. All other reagents were of the highest grade commercially available.

Cell growth and labelling

NBL-1 cells were grown routinely in Ham's F-12 medium supplemented with 10% (v/v) new-born calf serum, 100 units of penicillin/ml, 100 µg of streptomycin/ml, 2.5 µg of fungizone/ml and 2 mM-glutamine, at 37 °C in a water-jacketed incubator in an atmosphere of CO₂/air (1:19) [12].

For pulse-labelling of mitochondrial gene products, the method of Chomyn *et al.* [9] was used to a large extent, except that after growth in the presence of chloramphenicol [9], the cells were labelled in a low-methionine medium (1 in 20 dilution of Ham's F-12

Abbreviation used: URF, unidentified reading frame.

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medium with minus-methionine medium [11]) in the presence of cycloheximide (200 $\mu\text{g/ml}$). Integration of the gene products into functional NADH dehydrogenase was subsequently achieved by chasing the radiolabelled products in normal growth medium [9] at 37 °C for 17 h.

Subcellular fractionation of NBL-1 cells to give a particulate fraction enriched with mitochondria was performed using digitonin, essentially by the method of Mori *et al.* [13], as modified by Gibb & Ragan [11].

Immunoprecipitation from Triton X-100-solubilized material

For immunoprecipitation studies of assembled ND products, the particulate fraction $[(2-3) \times 10^6 \text{ d.p.m.}]$ derived from approx. 3×10^6 cells] was initially solubilized with 1 ml of NET buffer (0.15 M-NaCl/5 mM-EDTA/50 mM-Tris/HCl, pH 7.4) containing 0.5% (v/v) Triton X-100 and clarified by centrifugation at 20000 *g* for 25 min. A preliminary precaution that was undertaken when carrying out the immunoprecipitation reaction was to perform a non-specific immunoprecipitation by incubating the samples with 10 μl of pre-immune serum for 1 h at room temperature before the addition of 20 μl of a 10% (w/v) suspension of *S. aureus* cells. After a further 1 h of continual mixing, cells were collected by centrifugation for 1 min in an Eppendorf microfuge. The supernatant fraction was then treated with 10–30 μl of a specific antiserum and incubated for 3 h at room temperature. Immune complexes were collected in an identical manner to that described above. Cells were washed three times with NET buffer containing 0.5% (v/v) Triton X-100 and once with NET buffer alone. Immune complexes with ND products were recovered by treating the samples for 30 min at 37 °C and for 10 min at 50 °C in 50 μl of Laemmli [14] sample buffer. Proteins were resolved by SDS/gel electrophoresis on 15% (w/v) polyacrylamide gels [14] and revealed after treatment of the gel for fluorography [15].

Immunoprecipitation from SDS-treated material

For immunoprecipitation studies of dissociated ND products, the particulate fraction was solubilized in 1 ml of NET buffer containing 0.5% (v/v) Triton X-100 and clarified by centrifugation for 5 min in an Eppendorf bench centrifuge. The supernatant was diluted with an equal volume of NET buffer containing 0.5% (v/v) Triton X-100 and 0.4% (w/v) SDS and centrifuged for 20 min at 20000 *g*. Immunoprecipitation was carried out exactly as above.

Antibody production

Production of antisera to native Complex I and Complex III has been described previously [12,16]. Anti-ND-1 serum and anti-(cytochrome oxidase) serum were kindly donated to us by Professor G. Attardi (California Institute of Technology, Pasadena, CA, U.S.A.) and Professor J. B. Clark (Department of Biochemistry, St. Bartholomew's Hospital, London, U.K.) respectively.

RESULTS

Products of the bovine mitochondrial genome

Selective expression of the mitochondrial gene products in NBL-1 cells was achieved by radiolabelling of the cells with L-[^{35}S]methionine in the presence of the

reversible cytoplasmic protein synthesis inhibitor cycloheximide (200 $\mu\text{g/ml}$). On SDS gels, eleven polypeptides of apparent M_r values between 10000 and 51000 were consistently observed, and the pattern very closely resembled that found in HeLa cells [9,10] with respect to the number, apparent M_r and labelling intensity of the polypeptides. The eleven polypeptides are indicated in Fig. 1, track 2. Overexposure of the autoradiograph was required to reveal the smaller mitochondrial gene products, and the larger proteins can be seen more clearly against the background in Fig. 2, track 1. The background consists of high- M_r material, most of which fails to enter the gel. Although there are thirteen mitochondrial gene products, SDS/polyacrylamide gradient gels (as used in this paper) fail to resolve the two smallest proteins of HeLa cells, namely the ATPase 8 and ND-4L gene products. Thus the only qualitative difference between the results of Fig. 1, track 2 and those found with HeLa cells is the apparent absence of one product in the former of M_r in the range 20000–30000. Fig. 1, track 1 shows a Coomassie Blue-stained gel of bovine heart NADH dehydrogenase, which serves as an M_r marker track and also to illustrate that several of the mitochondrial gene products co-migrate with NADH dehydrogenase subunits (e.g. the 51000- M_r and 39000- M_r subunits). Fig. 1, track 3 shows that the synthesis of the mitochondrial gene products was inhibited by chloramphenicol (100 $\mu\text{g/ml}$). In the experiments with HeLa cells, identification of the bands on the gel with individual mitochondrial gene products was based on the use of antibodies. We have taken a similar approach with the bovine proteins.

Fig. 1. Mitochondrial gene products of NBL-1 cells

NBL-1 cells were pulse-labelled in the absence (track 2) or presence (track 3) of chloramphenicol (100 $\mu\text{g/ml}$), and mitochondrial fractions were analysed by SDS/gel electrophoresis and fluorography. Track 1 contained 50 μg of purified bovine heart Complex I, stained for protein. M_r values of some Complex I subunits are indicated. The putative mitochondrial gene products are indicated by dots to the right of track 2.

Fig. 2. Identification of mitochondrial gene products of NBL-1 cells

NBL-1 cells were pulse-labelled (track 1) or pulse-labelled and chased for 17 h (tracks 2–7). Track 1, total mitochondrial fraction; track 2, immunoprecipitate with anti-[Complex I (CI)] serum from Triton extract; track 3, immunoprecipitate with anti-[cytochrome oxidase (CO)] serum from Triton extract; track 4, immunoprecipitate with anti-[Complex III (CIII)] serum from Triton extract; track 5, as track 3, but from Triton/SDS extract; track 6, as track 4, but from Triton/SDS extract; track 7, immunoprecipitate with anti-ND-1 product serum from Triton/SDS extract. Samples were analysed by SDS/gel electrophoresis and fluorography. Identification of the proteins in track 1 was made as described in the text. The ND gene products are identified by dots to the right of track 2.

Identification of the bovine mitochondrial gene products

The ND gene products were identified by immunoprecipitation with an antiserum raised against native bovine heart Complex I. Labelled mitochondria were solubilized in 0.5% (w/v) Triton X-100 in which the structural integrity of NADH dehydrogenase is maintained, and immunoprecipitated material from the Triton extract was enriched in seven of the mitochondrial gene products as can be seen by comparing tracks 1 and 2 of Fig. 2. These seven proteins corresponded closely in apparent M_r with those identified as ND gene products in HeLa cells [9,10]. This conclusion was strengthened by the demonstration that several of the proteins not immunoprecipitated by antiserum to Complex I were components of other enzyme complexes. Thus two of these proteins were immunoprecipitated from a Triton X-100 extract by an antibody to cytochrome oxidase (Fig. 2, track 3). Only the smaller of these two was precipitated from an extract treated with SDS (Fig. 2, track 5). The antiserum contained antibodies directed against only one of the three mitochondrially encoded subunits, namely subunit III (J. B. Clark, personal com-

munication) which therefore must be the protein in track 5 of Fig. 2. The immunoprecipitate of the holoenzyme (track 3) showed only the two bands, the larger of which must be subunit I on the basis of its apparent M_r of approx. 45000. Subunit II was therefore not detected, or, more likely, was not resolved from subunit III in this gel system. The polypeptide of cytochrome *b* was identified by its precipitation from a Triton X-100 extract with an antiserum to Complex III (Fig. 2, track 4). Western blot analysis showed that this antiserum did not contain antibodies against cytochrome *b* and, therefore, as expected, precipitation from an SDS-treated extract did not give rise to the cytochrome *b* polypeptide (Fig. 2, track 6). The remaining polypeptide which was not precipitated by antiserum to Complex I was tentatively identified as the ATPase 6 subunit on the basis of its apparent M_r .

Of the seven polypeptides shown in Fig. 2, track 2, three could be positively identified. The ND-1 gene product was precipitated from an SDS-treated extract by a specific antiserum raised against the C-terminal region of the human protein (Fig. 2, track 7). This antiserum also reacted with a constituent of purified Complex I running at the same position on a gel, as shown by Western blotting [17]. Antisera raised to the C-terminal regions of the ND-3 and -4L products also cross-reacted with their bovine counterparts on Western blots [17], thereby providing positive identification of these two polypeptides. Immunoprecipitation experiments like those of Fig. 2 were not however successful with these two small proteins, perhaps because of sensitivity problems.

The remaining ND gene products (5, 4, 2 and 6) were assigned on the basis of their apparent M_r values and similarity with the human proteins. The rank order of apparent M_r (or migration on the gel) for these proteins and the other mitochondrial gene products is not dependent on the gel system used [9,10], with the exception of the cytochrome *b* polypeptide and subunit II of the cytochrome oxidase which were discussed above. Thus we are confident that the assignments shown in Fig. 2 are correct, even though definitive proof will require either the use of antibodies specific for the bovine proteins or isolation and partial sequencing of the appropriate polypeptide.

The apparent M_r values are compared with their actual M_r values in Table 1. There is reasonably good

Table 1. Apparent M_r values of ND gene products

ND product	Theoretical M_r	Apparent M_r
1	35675	30000
2	39260	33000
3	13056	13600
4	52107	39000
4L	10799	10500
5	68323	51000
6	19080	19000

Theoretical M_r values were derived from the bovine mitochondrial gene sequence [3], whereas apparent M_r values were derived by SDS/gel electrophoresis as described in the Materials and methods section.

agreement between the two for the ND-1, -6, -3 and -4L products. However, the putative ND-5, -4 and -2 products migrated anomalously with apparent M_r values of 51 000, 39 000 and 33 000 respectively.

The largest ND gene product (tentatively identified as ND-5) migrates on SDS gels indistinguishably from the 51 000- M_r subunit of Complex I (Fig. 1), a component of the flavoprotein fragment of the enzyme [8]. The flavoprotein subunit of this M_r has been shown to be nuclear-encoded by the sensitivity of its synthesis to cytoplasmic protein synthesis inhibitors [16] and by the fact that the protein is synthesized as an M_r -52 000 cytoplasmic precursor in the presence of uncoupling agents [12]. The amino acid composition of the flavoprotein subunit is also inconsistent with its being the ND-5 gene product [16]. We conclude therefore that NADH dehydrogenase contains two subunits of this apparent M_r , one which is nuclear-encoded and one which is mitochondrially encoded. This conclusion is of relevance to previous work on the binding site of the inhibitor rotenone [17]. Photolabelling of isolated NADH dehydrogenase with [3 H]dihydrorotenone gave rise to incorporation of radioactivity into the ND-1 gene product and an additional polypeptide of apparent M_r 51 000. It is extremely unlikely that the 51 000- M_r flavoprotein subunit is involved in specific binding of rotenone [8], and a better explanation is that it was the putative ND-5 gene product which was labelled.

The ND-4 product (true M_r 52 000) appears to be the subunit of NADH dehydrogenase of apparent M_r 39 000 (Figs. 1 and 2), a component of the hydrophobic fraction of the enzyme [8]. Attempts to immunoprecipitate this subunit from SDS-treated extracts with a monospecific antiserum to the bovine 39 000- M_r subunit [18] were not successful, and definitive identification will require sequencing of this protein.

The subunits of the enzyme with M_r values around 30 000 have proved awkward to resolve by SDS/gel electrophoresis, since their apparent M_r varies with the gel composition. The ND-1 product (M_r 30 000 in this study) can co-migrate with the 30 000- M_r subunit of the iron-protein fragment [12], but in some gel systems it migrates more slowly and has previously been referred to as the 33 000- M_r subunit [8]. From the present study, the ND-2 product migrates even more slowly (apparent M_r 33 000 in Figs. 1 and 2), but frequently does not stain heavily and had not previously been clearly identified in work from this laboratory, although a subunit co-migrating with the ND-2 product can be seen in Fig. 1, track 1. In a recent paper, Yagi & Hatefi [19] reported the

presence of three polypeptides in this apparent size range in preparations of the hydrophobic protein fragment of bovine NADH dehydrogenase, the smallest of which was the ND-1 product.

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