NADH:ubiquinone oxidoreductase from bovine heart mitochondria: sequence of a novel 17.2-kDa subunit

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Abstract The sequences of 41 subunits of complex I (NADH:ubiquinone oxidoreductase) from bovine heart mitochondria have been described previously. Seven of them are encoded in mitochondrial DNA, and the remainder are nuclear gene products that are imported into the organelle from the cytoplasm. By electrospray mass spectrometry experiments conducted on complex I and on two related subcomplexes, an additional protein has been identified with a mass not corresponding to any of the known subunits of the enzyme. This protein has also been found in samples of the enzyme fractionated on two dimensional polyacrylamide gels. Material from these gels has been digested with trypsin and peptide sequences have been determined, confirming that the protein did not correspond to any of the known subunits of complex I. The cDNA sequence of this protein, determined with the aid of the peptide sequences, demonstrates that it is a novel subunit of complex I, and that it is related to a 13-kDa human protein associated with differentiation.

Key words: Mitochondrion; Complex I; New subunit; Sequence

1. Introduction

Complex I (NADH:ubiquinone oxidoreductase) is found in the inner membranes of mitochondria and eubacteria, and provides the entry point for electrons from NADH into their electron transport chains. The enzyme from bovine heart mitochondria is an assembly of at least 41 different proteins that contains one molecule of non-covalently bound FMN and many Fe-S clusters [1]. Seven of its subunits are products of the mitochondrial genome, and the remainder are nuclear gene products that are imported into the organelle. These various components have a combined molecular mass of about 900 kDa, and because it is so large, the individual assemblies can be visualized readily by electron microscopy. Complex I is L-shaped, with one arm in the lipid bilayer and the other protruding from it. The protruding globular arm appears to become narrower close to the membrane surface [2,3]. It can be dissociated from the membrane domain as a water soluble assembly known as subcomplex Iλ that can transfer electrons from NADH to artificial acceptors such as ferricyanide, but not to quinones [4]. Subcomplex Iλ contains about 14 of the subunits of complex I, all of the known Fe-S clusters, the non-covalently bound FMN prosthetic group, and the NADH binding site [4,5].

By electrospray mass spectrometry experiments conducted on complex I and on two related subcomplexes, a protein has been identified with a mass not corresponding to any of the known subunits of bovine complex I. The same protein has been found in bovine complex I fractionated on two-dimensional polyacrylamide gels. The sequences of tryptic peptides were used to design synthetic mixed oligonucleotides for use in polymerase chain reactions (PCRs) that led to complete cDNA and protein sequences. The mature protein sequence of the subunit, designated B17.2, contains 145 amino acids, and has an acetylated α-amino group. Its sequence is related to a human 13-kDa differentiation associated protein.

2. Materials and methods

2.1. Materials

Complex I was purified from bovine heart mitochondria [6]. Subcomplexes Iα and Iλ were prepared from complex I [4,5]. Trypsin (sequencing grade) was obtained from Boehringer Mannheim (Lewes, UK). Aquapore and Poros R2 reverse phase chromatography columns were supplied by Applied Biosystems, Warrington, UK and PerSeptive Biosystems, Hertford, UK, respectively. Oligonucleotides were synthesized with an Applied Biosystems 380B instrument.

2.2. Reverse-phase high pressure liquid chromatography of subunit B17.2

Complex I, and subcomplexes Iα and Iλ, were precipitated from dodecyl maltoside solutions with 20-fold excesses (v/v) of ice-cold ethanol. The suspensions were kept for 16 h at −20°C. The precipitates were redissolved in 6 M guanidinium hydrochloride in 0.1% (v/v) trifluoroacetic acid, and the subunits were fractionated by reverse-phase high pressure liquid chromatography (HPLC). Chromatography was conducted either on an Aquapore C8 column (300 Å pores, 7 μ particles, 100×2.1 mm i.d.), with a Hewlett Packard 1090M liquid chromatograph, or on a Vydac C8 column (150×1.0 mm i.d.) with an Ultrafast Microprotein Analyzer (Michrom BioResources, Auburn, CA, USA). Both columns were equilibrated in 0.1% (v/v) trifluoroacetic acid, and developed with a linear gradient of acetonitrile containing 0.09% (v/v) trifluoroacetic acid. Portions of eluates were examined by electrospray ionization mass spectrometry (ESI-MS), either 'off line', (from 2-mm i.d. columns), or 'on-line' (from 1-mm i.d. columns) [7].

2.3. Two dimensional gel analysis

The subunits of complex I were separated on two dimensional polyacrylamide gels [8]. The first dimension (NEPHGE) was conducted in tube gels (170×0.75 mm i.d.) containing 2% ampholines (1.5%, pH range 7–9, and 0.5%, pH range 3.5–10). Samples (40 μg) dissolved in a solution (10 μl) containing 9.5 M urea, 2% Nonidet NP40, and 2% ampholines (1.5%, pH 7–9, 0.5%, pH 3.5–10) were applied at the anode end. The reservoirs contained 0.2% (v/v) orthophosphoric acid (anode), and 0.4% (v/v) 1,2-diaminoethane (cathode). A potential of 400 V was applied for 6 h. Then the gel was extruded into a buffer

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Abbreviations: ESI-MS, electrospray ionization mass spectrometry; HPLC, high pressure liquid chromatography; NEPHGE, non-equilibrium pH gel electrophoresis; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction

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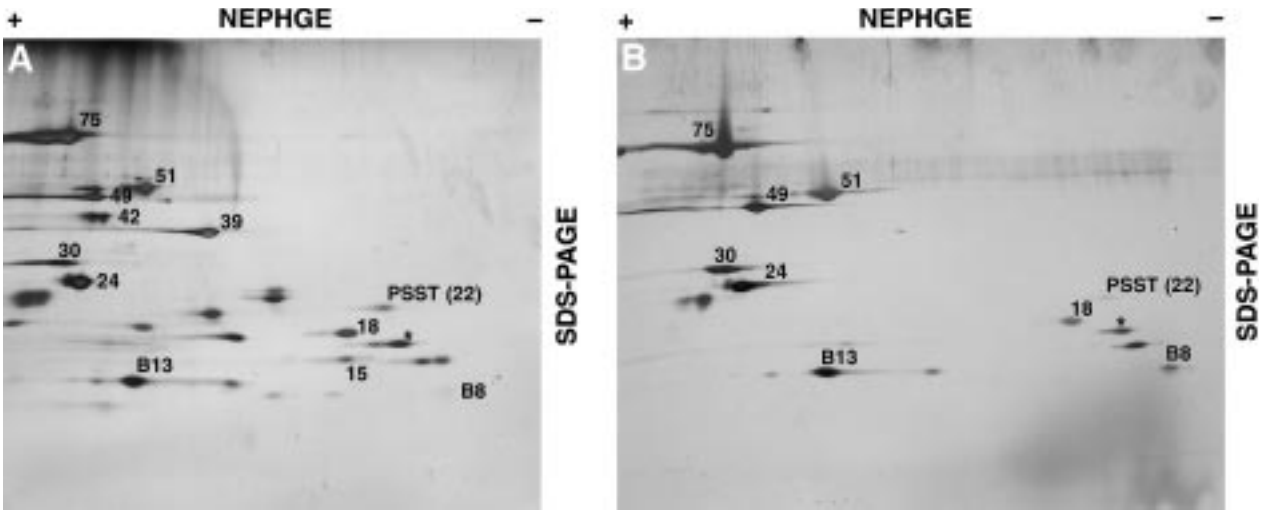

Fig. 1. Separation of the subunits of bovine mitochondrial complex I and subcomplex Iλ on two dimensional gels by NEPHGE and SDS-PAGE. A: Complex I; B: sub-complex Iλ. The proteins were detected by silver staining. Subunits identified by protein chemical means (see Table 1) are indicated by their estimated molecular masses. The position of subunit B17.2 is indicated by an asterisk.

containing 0.06 M Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol, and 10% glycerol. The gel was shaken in this solution for 20 min and then placed on a 10–25% polyacrylamide gradient slab Laemmli gel (20×20 cm×0.75 mm) [9]. The second dimension (SDS-polyacrylamide gel electrophoresis (PAGE)) was developed until the bromophenol blue marker dye had reached the bottom of the gel.

Subunits of complex I were transferred from two dimensional gels onto poly-(vinylidene-difluoride) membranes [10], and proteins were detected with PAGE blue 83 dye. Proteins in gels were stained either with PAGE blue 83 dye, or silver [11], development being terminated with 5% acetic acid. Spots were excised for further analysis.

2.4. Enzymic digestion of subunits and purification of peptides

Tryptic digestions were performed as described before [11], except that gel pieces containing the B17.2 subunit were first macerated and then digested in Tris-HCl buffer (20 mM, pH 8.0). Two tryptic digests of subunit B17.2 from identical blue stained gels were combined and fractionated by reverse phase HPLC on an Aquapore C18 column (100×2.1 mm i.d.) equilibrated in 0.1% (v/v) trifluoroacetic acid. Purified peptides were sequenced in an Applied Biosystems Procise 494 instrument.

An unfractionated tryptic digest of subunit B17.2 obtained from a single silver stained spot from a two dimensional gel was freed of salts and other impurities on Poros R2 (20 Å pores) in a glass capillary similar to a nanoelectrospray needle (bed volume, 50–100 nl) [11]. Peptides were applied to the column in 5% aqueous methanol containing 5% formic acid (10 μl), and the column was washed twice with the same solvent (3 μl). Peptides were eluted with 60% aqueous methanol containing 5% formic acid (total 1 μl) and transferred directly to a nanoelectrospray needle.

2.5. Mass spectrometry

The molecular masses of subunits of complex I [7,12] were measured in a Perkin Elmer Sciex API III$^+$ triple quadrupole mass spectrometer with pneumatically assisted electrospray ionization. Tandem mass spectrometry experiments were conducted in the same instrument fitted with a nanoelectrospray source [13], which allowed 1-μl samples of a tryptic digest of subunit B17.2 to be analyzed for up to 1 h, and for multiple mass measurements to be recorded. First, spectra were recorded on the digest. Then peptide ions were distinguished from those arising from non-peptide contaminants by subjecting each ion in turn to collisional fragmentation with argon. The resultant spectra were examined for the presence of immonium ions (*m*/*z* 86) which arise from isoleucine and leucine side chains and so are diagnostic of peptide ions [14]. Then each peptide ion was selected, fragmented and its fragment ion tandem mass spectrum recorded. Partial amino acid sequences were deduced from the tandem mass spectra.

2.6. Amplification and sequencing the cDNA for subunit B17.2

The amplification of the cDNA encoding the B17.2-kDa subunit of bovine complex I was carried out as described previously [15,16]. First, mixtures of synthetic oligonucleotides based on the partial tryptic peptide sequences KFNLSG and IQEWVP were used as primers in a PCR with total bovine cDNA as template. The partial cDNA bridging these two sequences was detected in the reaction products by hybridization with a third mixture of oligonucleotides based on the sequence QQYVPY. In two further PCRs with unique primers (2F, 2R and 3R, see Fig. 2) and with oligo dT primers in the opposite sense, the partial cDNA was sequenced and then extended to the 5′ and 3′ extremities of the intact cDNA. For the 5′ extension, the template was bovine heart cDNA with a 5′ poly(A) tail added with terminal transferase [16]. DNA sequences were determined by the modified dideoxy method [17].

3. Results and discussion

3.1. Evidence for a new subunit of complex I

During the characterization of the subunit compositions of bovine complex I and subcomplexes Iα and Iλ, a component

Table 1
Identification of subunits of complex I from bovine mitochondria

Subunit	Method of identification	
	Complex I	Subcomplex Iλ
75 kDa	1	1
51 kDa	1, 2	1
49 kDa	1, 2	1
42 kDa	1	Not present
39 kDa	1, 2	Not present
30 kDa	1, 2	1
24 kDa	1, 3	1
PSST (22)	1, 2	1
18 kDa	1, 2, 3	1
B17.2	2, 3	4
15	1, 2, 3	Not present
B13	2, 3	4
B8	3	4

Subunits of complex I and subcomplex Iλ were separated on two-dimensional gels (see Fig. 1) and identified by various methods. 1: By N-terminal sequence analysis; 2: by tandem MS of tryptic peptides; 3: by Edman degradation of tryptic peptides; 4: by reference to gel in Fig. 1A.

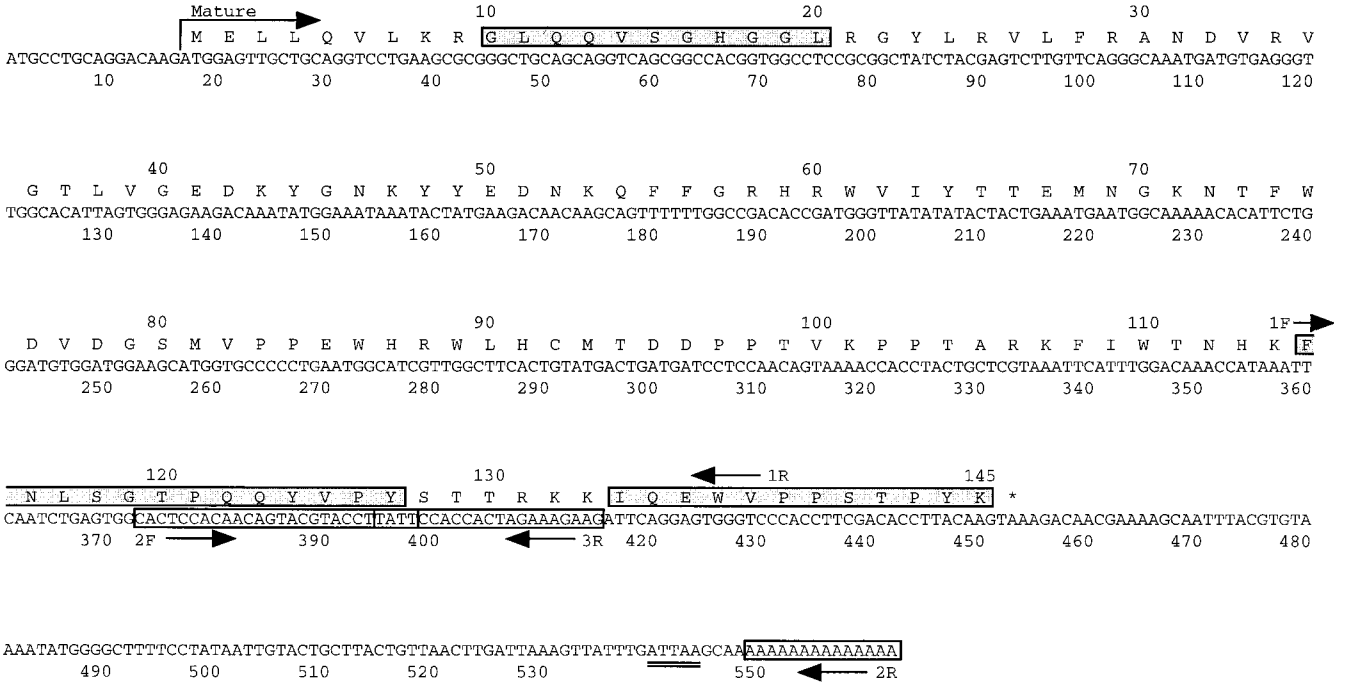

Fig. 2. Sequence of the cDNA encoding the B17.2 subunit of complex I from bovine mitochondria. The shaded regions were determined by sequencing tryptic peptides. The boxed sequences 1F and 1R were used to design synthetic oligonucleotide mixtures employed in an initial PCR. The resulting partial cDNA was extended to the 3′ and 5′ extremities with primers based on the two boxed nucleotide sequences 2F and 3R and primers complementary to poly(A) and poly(G) sequences at the 3′ and 5′ ends [16]. The senses of the primers are indicated by arrows. A polyadenylation signal is underlined twice.

with a mass of 17 208 ± 2 was observed by ESI-MS on a number of occasions. Often it was accompanied by another abundant component with a mass of 17 131. Neither of these masses corresponds to any of the 41 characterized subunits of the enzyme. Therefore, they appeared to represent new subunits of the enzyme. It is now evident from the data described below that the mass of 17 131 corresponds to the α-*N*-acetylated B17.2 subunit with a reduced cysteine residue, and that the 17 208 ± 2 value arises from the same B17.2 protein with an artefactual mercaptoethanol (+76 mass units), attached by a disulfide linkage. Mercaptoethanol adducts have been detected before during the characterization of other complex I subunits, particularly from subunit B13 [15].

Subunit B17.2 co-eluted from C18 HPLC columns with subunits B14, B13 and MLRQ [15] at 46% acetonitrile. No new N-terminal sequences were found in these fractions by Edman degradation, and so subunit B17.2 appeared to have a modified N-terminus, as have 18 other subunits of the complex [15,18]. Subunit B17.2 could not be separated from these other subunits by chromatography, and so it was purified by two dimensional electrophoresis of complex I and its subcomplexes. Many subunits of bovine complex I have apparent molecular weights in the range between 10–20 kDa and are basic. Therefore, it was necessary to combine SDS-PAGE with NEPHGE. As summarized in Fig. 1 and Table 1, various subunits were identified on such gels by protein chemical methods. One spot (labelled with an asterisk), gave rise to tryptic peptide sequences that did not belong to any of the known subunits of the complex. This spot contains subunit B17.2.

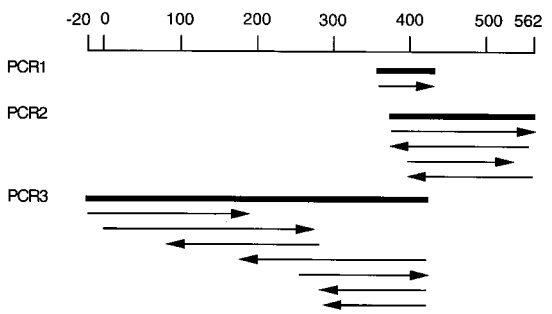

Fig. 3. Generation by PCR and sequence analysis of the cDNA clones encoding the B17.2 subunit of complex I from bovine heart mitochondria. PCRs 1–3 denote the cloned partial cDNAs generated by PCRs. The heavy lines represent the cDNA fragments, and the arrowed lines indicate the directions and extents of the determined sequences.

3.2. The cDNA sequence of the new subunit of complex I

Three sequences obtained by Edman degradation of tryptic peptides (see Fig. 2) were used as the basis for isolating a partial cDNA from bovine cDNA (see Section 2, and legends to Figs. 2 and 3). In two further PCRs, this partial cDNA sequence was extended to the 5′ and 3′ extremities of the intact cDNA (see Figs. 2 and 3). The 5′ end of the cDNA sequence (Fig. 2) terminates close to the proposed initiation codon, and does not contain an up-stream termination codon in phase. The protein mass calculated from the protein sequence is 17 131.6 Da, assuming that the N-terminal methionine is acetylated in the mature protein. This value corresponds to the mass measured by ESI-MS (17 130.8 Da). The hydrophobic profile (not shown) indicates that the protein is hydrophilic and has no extensive hydrophobic sequences. These properties are consistent with the subunit being associated with subcomplex Iλ which represents the globular extramembrane domain of complex I. Subcomplex Iα contains all

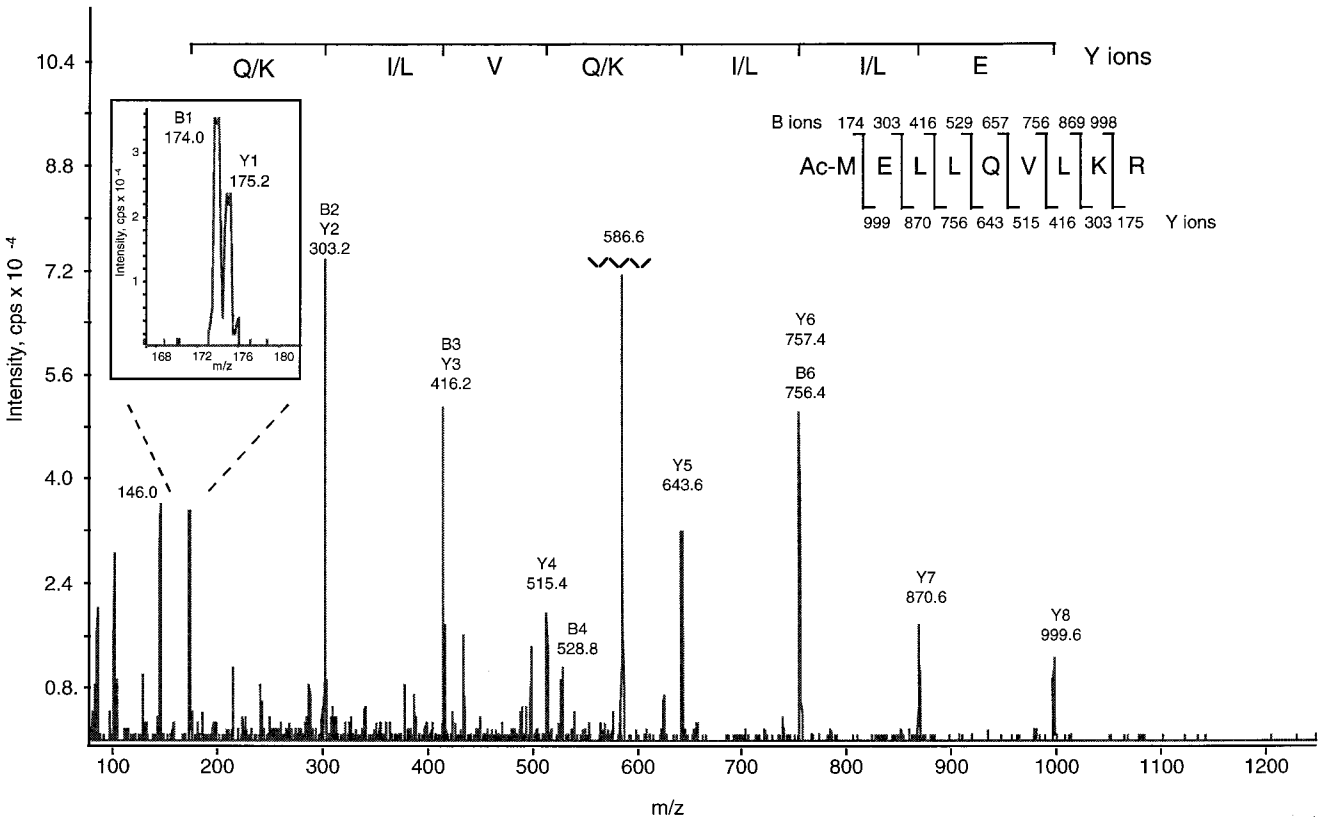

Fig. 4. Fragment ion spectrum obtained by tandem mass spectrometry of the N-terminal tryptic peptide of subunit B17.2 of bovine complex I. A doubly charged ion (*m/z* 586.6) was selected and collided with argon gas with a collision energy of 40 eV. The inset contains an expanded region of the spectrum from *m/z* 168–180. The Y fragment ions [19] were interpreted as a partial amino acid sequence, as indicated. The predicted nominal masses of B and Y ions are also indicated. Ac-M is *N*-acetylmethionine.

the subunits in subcomplex Iλ and four additional hydrophobic subunits. Subunit B17.2 is the 18th nuclear coded subunit of complex I that evidently lacks a cleavable presequence to direct its import into the mitochondrion.

3.3. The N-terminus of subunit B17.2 is acetylated

The *m/z* values of two ions (586.6 and 594.6) observed in tandem mass spectrometry (MS) experiments on tryptic peptides of B17.2 correspond to forms of the N-terminal tryptic peptide (Ac-MELLQVLKR), with reduced and oxidized methionines, respectively. From the fragment ion spectrum of a doubly charged ion of *m/z* 586.6 (Fig. 4), the amino acid sequence E-I/L-I/L-Q/K-V-I/L-Q/K, R was deduced from a series of Y ion fragments [19], demonstrating that the peptide is the N-terminal fragment of B17.2. Its mass (MH^+ 1172.2), and the fragment ions B1 (*m/z* 174) and B2 (*m/z* 303), corresponding to Ac-M and Ac-ME, show that the N-terminal methionine is acetylated. An expanded region of the fragment ion (see inset in Fig. 4) distinguishes the B1 ion (*m/z* 174) from the C-terminal arginine Y1 ion (*m/z* 175).

3.4. Homology of bovine B17.2 with a human protein

Residues 70–132 of subunit B17.2 of bovine complex I are 70% identical to a human 13-kDa protein, in the NCBI non-

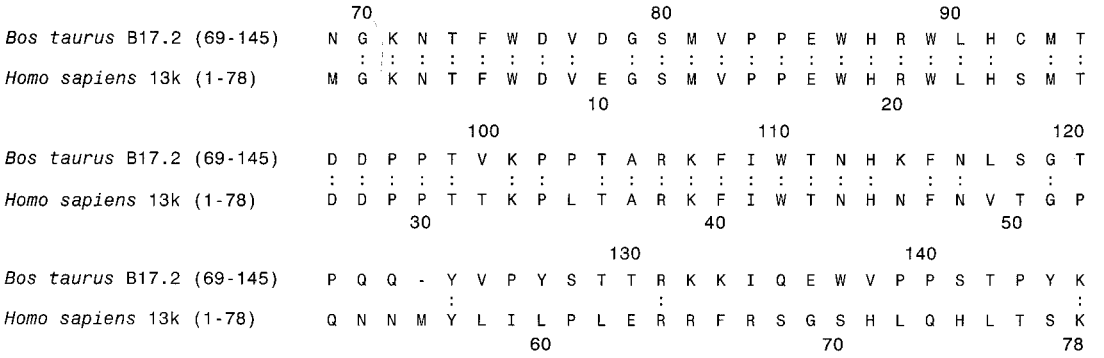

Fig. 5. Alignment of the sequences of the B17.2 subunit of bovine complex I and a 13-kDa human 'differentiation protein'. Colons denote residues that are identical in both sequences.

redundant database (accession number 995939; see Fig. 5). Little is known about the human protein, except that it is described as a theoretical product of a gene identified in an adenocarcinoma cell line which had been induced to differentiate by treatment with all *trans*-retinoic acid. For this reason, the gene and its product are described as being 'differentiation associated'. It is possible that the 13-kDa protein is part of the B17.2 kDa homologue in human complex I.

3.5. Subunit composition of complex I

The B17.2 subunit is the 42nd subunit to be sequenced from bovine mitochondrial complex I. From time to time, another component with a molecular mass of $10\,566 \pm 2$ Da has been observed in ESI-MS experiments carried out on complex I and subcomplex Iβ [12]. This protein has not been sequenced yet, but it is likely to be the 43rd and final subunit of the complex.

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