Characterization of Two cDNA Clones for Pyruvate Dehydrogenase E1β Subunit and Its Regulation in Tricarboxylic Acid Cycle-deficient Fibroblast*

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Two distinct types of cDNA clones encoding for the pyruvate dehydrogenase (PDH) E1β subunit were isolated from a human liver λgt11 cDNA library and characterized. These cDNA clones have identical nucleotide sequences for PDH E1β protein coding region but differ in their lengths and in the sequences of their 3' -untranslated regions. The smaller cDNA had an unusual polyadenylation signal within its protein coding region. The cDNA-deduced protein of PDH E1β subunit revealed a precursor protein of 359 amino acid residues (M, 39,223) and a mature protein of 329 residues (M, 35,894), respectively. Both cDNAs shared high amino acid sequence similarity with that isolated from human foreskin (Koike, K. K., Ohta, S., Urata, Y., Kagawa, Y., and Koike, M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 41-45) except for three regions of frameshift mutation. These changes led to dramatic alterations in the local net charges and predicted protein conformation. One of the different sequences in the protein coding region of liver cDNA (nucleotide position 452-752) reported here was confirmed by sequencing the region after amplification of cDNA prepared from human skin fibroblasts by the polymerase chain reaction. Southern blot analysis verified simple patterns of hybridization with E1β cDNA, indicating that the PDH E1β subunit gene is not a member of a multigene family. The mechanisms of differential expression of the PDH E1α and E1β subunits were also studied in established fibroblast cell lines obtained from patients with Leigh's syndrome and other forms of congenital lactic acidosis. In Northern blot analyses for PDH E1α and E1β subunits, no apparent differences were observed between two Leigh's syndrome and the control fibroblasts studied: one species of PDH E1α mRNA and three species of E1β mRNA were observed in all the cell lines examined. However, in one tricarboxylic acid cycle deficient fibroblast cell line, which has one-tenth of the normal enzyme activity, the levels of immunoreactive PDH E1α and E1β subunits were markedly decreased as assessed by immunoblot analyses. These data indicated a regulatory mutation caused by either inefficient translation of E1α and E1β mRNAs into protein or rapid degradation of both subunits upon translation. In contrast, the PDH E1α and E1β subunits in two fibroblast cell lines from Leigh's syndrome patients appeared to be normal as judged by 1) enzyme activity, 2) mRNA Northern blot, 3) genomic DNA Southern blot, and 4) immunoblot analyses indicating that the lactic acidosis seen in these patients did not result from a single defect in either of these E1α and E1β subunits of the PDH complex.

The pyruvate dehydrogenase (PDH) complex catalyzes the oxidative decarboxylation of pyruvate producing acetyl coenzyme A, CO2, and NADH. Pyruvate dehydrogenase complex consists of pyruvate dehydrogenase (E1) (EC 1.2.4.1), dihydrolipoyl transacetylase (E2) (EC 2.3.1.12) and dihydrolipoamide dehydrogenase (E3) (EC 1.8.1.4) (for review see Ref. 1). Pyruvate dehydrogenase activity is thought to be regulated via phosphorylation and dephosphorylation by a specific kinase (EC 2.7.1.99) and a specific phosphatase (EC 3.1.3.43), respectively (2-4). Because of the central role of the PDH complex in glucose metabolism and energy production, this enzyme complex has been extensively studied in a variety of pathological conditions as to structure, function, subunit interaction, and regulation of enzyme activity (1, 4, 5). Defects in any one of the components may result in congenital lactic acidosis, which manifests symptoms varying from mild to severe ataxia (6, 8), or in a form designated as Leigh's encephalomyelopathy, which is characterized by mental and growth retardation with occasional premature death (9, 10). These diseases usually follow an autosomal recessive pattern of inheritance (5, 11). It has been suggested that 2,3-butanediol, found in the serum of human alcoholics both in the presence (12-14) and the absence of ingested ethanol (15), may result from the reduction of acetoin, a reaction product of the PDH E1 subunit (16-18).

Despite numerous studies of the catalytic properties and the regulation of the PDH E1α subunit, E1β subunit has not been well characterized regarding its structure and functional interaction with the PDH E1α subunit. Recent data suggested that a deficiency of the E1α and E1β subunits may be responsible for certain forms of lactic acidosis. (19). Here we report the complete nucleotide and deduced protein sequences of two cDNAs encoding for the human liver PDH E1β subunit. We also present the evidence of differential regulation of the PDH E1α and E1β subunits in cultured fibroblast cell lines including two cell lines from Leigh's syndrome patients and one cell line from a patient deficient in tricarboxylic acid cycle.

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The abbreviations used are: PDH E1α and E1β, pyruvate dehydrogenase E1α and E1β subunits; kb, kilobase pairs; SDS, sodium dodecyl sulfate.
Reagents—The restriction enzymes, Tt DNA ligase, polynucleotide kinase, and molecular weight standards (for protein, DNA, and RNA) were purchased from Bethesda Research Laboratories. The random primed labeling kit and dideoxy DNA sequencing kits with Sequenase were the products of Boehringer Mannheim and United States Biochemical Corporation (Cleveland, OH), respectively. [α-32P]dCTP (300 Ci/mmol), [γ-32P]ATP (3000 Ci/mmol), [α-32P]dATP (500 Ci/mmol), and GeneScreen membrane were obtained from Du Pont—New England Nuclear.

Isolation and Sequencing of cDNA Clones—A 4011 human liver cDNA library (kindly provided by Dr. Frank Gonzalez, National Cancer Institute) was screened with the oligodeoxynucleotide probe labeled with γ-32P]ATP by Tt polynucleotide kinase. The conditions of hybridization and subsequent washing were the same as described by Davis et al. (12). Positive plaques were identified by endonuclease digestion. The plasmid DNA was purified and amplified by the plate lysate method (22). The isolated DNA inserts were subcloned into plasmid pUC13 and subjected to nucleotide sequencing. A cDNA insert, with highly homologous nucleotide sequence to that of foreskin (20), was labeled with [α-32P]dCTP by random primed labeling kit. This cDNA insert was used as a probe for further screening of the cDNA library and the full-length cDNA clone was achieved by hybridization with [32P]-labeled cDNA probe and subsequent plaque purification.

Plaque Purification and DNA Sequencing—Large cDNA clones were then characterized by restriction endonuclease mapping and nucleotide sequencing. DNA sequencing was performed by dye-oligonucleotide-chain termination method (23) using M13mp18 and M13mp19 single strand vectors with modified Tt DNA polymerase system. Sequence data were assembled and analyzed by the Microgenie software program (Beckman Instrument, Menlo Park, CA).

Cell Culture of Skin Fibroblasts—Four established skin fibroblast cell lines (age and sex matched) were obtained from Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research, Camden, NJ. These cell lines include one fibroblast cell line (GM1654) with apparently normal level of PDH activity (which was used as the control), two from Leigh’s syndrome patients (GM1503 and GM3672), and one from a patient deficient in the tricarboxylic acid cycle (GM3093). These cells were cultured by the method of Sheu et al. (13) and one from a patient deficient in the tricarboxylic acid cycle (GM3093). These cells were cultured by the method of Sheu et al. (13)

Synthesis of Oligodeoxynucleotide Probe—Based on the published nucleotide and deduced protein sequences of human pyruvate dehydrogenase Eβ subunit (20), a oligodeoxynucleotide was synthesized by the phosphoramidite method using a DNA synthesizer (Codel 300, Du Pont). The synthesized probe near the amino-terminal was Asp-Glu-Glu-Leu-Glu-Arg-Asp (5′-GATGAGGAGCTGGAAAGAGAT-3′) prepared at 20% phosphoramidite.

Cellular DNA-Cell confluent cells (6-8 days after passage) in 250 ml culture flasks were washed three times with ice-cold phosphate-buffered saline. Cells were then collected either by scraping with rubber policeman or trypsinization depending on the purposes of the experiments.

Preparation of PDH Eα Subunit—Confluent cells (6-8 days after passage) in 250 ml culture flasks were washed three times with Hank's balanced salt media (GIBCO) and then harvested with 0.05% trypsin and 50 mM EDTA in Hank's balanced media. After a 10 min incubation at room temperature, cells were resuspended in 5 ml of Dulbecco's phosphate-buffered saline, pH 7.3. Cells were centrifuged at 12,000 g for 4 min, washed again with phosphate-buffered saline, and again collected by centrifugation. Cells were suspended in 0.3 ml of phosphate-buffered saline, pH 7.3, containing 5 mM dichloroacetic acid, and then incubated at 37 °C in a shaking incubator. After 15 min, 0.1 ml of a solution, pH 7.8, containing 40% ethanol, 25 mM sodium fluoride (NaF), 25 mM EDTA, and 4 mM dithiothreitol, was added (11). Cells were gently mixed and immediately frozen in liquid nitrogen and stored overnight at -80 °C. The next morning cells were frozen and thawed twice, immediately prior to assay.

PDH Activity—PDH activity in crude cell extracts was assayed by following acetol coenzyme A production at 37 °C as the method described by Cho and Hsu (24) using 2-oxoacetyl coenzyme A-arylamylamine N-acetyltransferase used in the PDH assays was prepared from pigeon liver aceacetate polypeptide (Sigma). Extraction and acetone precipitation were done as described by Tabor et al. (25). Enzymatic activity was then precipitated by 40-60% saturated ammonium sulfate. The precipitate was collected by centrifugation, dissolved in a minimal volume of 20 mM potassium phosphate buffer, pH 7.6, containing 5 mM 2-mercaptoethanol, and dialyzed against the same buffer. The dialyzed was loaded on to a DE52 column equilibrated with 20 mM potassium phosphate buffer, pH 7.6, containing 5 mM 2-mercaptoethanol. The column was then washed with the same buffer and enzymatic activity was eluted with a 0-0.5 M NaCl gradient in a 20 mM potassium phosphate buffer, pH 7.6, containing 5 mM 2-mercaptoethanol. Fractions with activity were pooled and again concentrated by precipitation with 40-60% ammonium sulfate. The precipitate was dissolved in a minimal volume, dialyzed overnight in 20 mM potassium phosphate buffer, pH 7.6, containing 5 mM 2-mercaptoethanol, and frozen at -20 °C until assayed. Under the conditions used pyruvate dehydrogenase activity was measured with respect to the amount of enzyme added to the assay mixture. Protein concentration was determined using Peterson’s modification (26) of the method of Lowry et al. (27).

Immunopurification of Rabbit Polyclonal Antibodies—Bovine kidney pyruvate dehydrogenase complex (specific activity of 14.4 units/mg protein at 30 °C) was purified according to the methods of Pettit and Rees (28). The purified PDH Eα and Eβ subunits isolated from the bovine kidney PDH complex were used to prepare polyclonal antibodies in rabbits which were subsequently purified before use by the method described below. PDH Eα and Eβ subunits were further separated by preparative SDS-polyacrylamide gel electrophoresis by the method of Laemmli (29) and then electrophoretically transferred to Immobilon-polyvinylidene difluoride membrane (Millipore Company, Bedford, MA) using carbonate transfer buffer (10 mM sodium bicarbonate, 3 mM sodium carbonate, pH 10) containing 20% methanol. The membrane strips corresponding to PDH Eα and Eβ subunits were carefully excised and incubated with respective rabbit polyclonal antibodies against bovine kidney PDH Eα and Eβ subunits. Following removal of nonspecifically bound materials to membrane strips by washing with 0.1 M potassium phosphate buffer, pH 4.5, containing 1.0 M NaCl, the bound specific polyclonal antibodies against Eα and Eβ subunits were eluted from the membrane strips with small volumes of 0.1 M glycine-HCl buffer, pH 3.0, and immediately neutralized by the addition of 40 μl of 1 sodium, 1 ml of glycine buffer. Purified specific rabbit antibodies against respective proteolytic PDH Eα and Eβ subunits were then used for immunoblot analyses.

Genomic DNA Southern and mRNA Northern Blot Analyses—Genomic DNA (10 μg) isolated from cultured fibroblasts by the method of Davis et al. (21) was digested with EcoRI, BamHI, HindIII and PstI at 37 °C overnight and separated on 0.8% agarose gel. Transfer of the DNA fragments to GeneScreen membrane, prehybridization, and Southern blot hybridization were performed with a 32P-labeled cDNA probe as described by Maniatis et al. (22). Northern blot analyses were performed using total cytosolic RNA isolated from human skin fibroblasts by the method of Chirgwin et al. (30). Total cytosolic RNAs (10 μg) were electrophoresed on 2.5 M formaldehyde, transferred to GeneScreen membrane, and hybridized with a [32P] cDNA probe as described by Maniatis et al. (22). Both prehybridization and hybridization were processed at 60 °C overnight in a 6 X SSC (0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0) solution containing 5 × Denhardt’s solution, 100 μg/ml salmon sperm DNA, and 0.1% SDS. After hybridization with 32P-labeled cDNA probes, the membranes were washed at 30 °C for four to five times with 2 × SSC containing 1.0% SDS. If needed, a second series of washing with 0.2 × SSC containing 0.1% SDS was performed. The exact conditions of washing were empirically determined by comparing the positive signals with background labeling. X-ray films (Eastman Kodak) were exposed to the GeneScreen membranes with the exposed films being negativized.

Immunoblot Analysis—The whole homogenates of cultured fibroblasts (100 μg) were separated on 10% SDS-polyacrylamide gel, transferred to Immobilon-polyvinylidene difluoride membranes which were subsequently blocked with 3% (w/v) non-fat dry milk dissolved in phosphate-buffered saline for 30 min at room temperature prior to immunoblotting. The primary antibodies used were the same as those used for the immunopurification of rabbit polyclonal antibodies against PDH Eα and Eβ subunits. The bound primary antibodies were further recognized by incubation with a secondary goat-anti rabbit IgG conjugated with alkaline phosphatase. This was followed by the color development using 5-bromo-4-chloro-3-indolyl phosphate combined with nitro blue tetrazolium (Kirkegaard and Perry Laboratory, Gaithersburg, MD) as chromogenic substrates.

Polymerase Chain Reaction—Total cytosolic RNA isolated from normal human skin fibroblasts by the method of Chirgwin et al. (30) was further purified by using (oligo)DT column (21) to isolate the
poly(A) RNA. Collected mRNA was converted to cDNAs using cDNA synthesis kit from Boehringer Mannheim in the manufacturer's protocol. PDH Eβ cDNA, in newly synthesized total cDNA mixtures prepared from the poly(A)+ RNA of human skin fibroblasts, was amplified by the polymerase chain reaction with synthesized oligodeoxynucleotide primers: primer 1, 5'-CACGAGTGTAGCCTGCCCCAGT-3' (nucleotide position 452-471 in Fig. 2) and primer 2, 5'-TTAAACGAGCTCCTGCCTGC-3' (antisense of nucleotide position 752-732 in Fig. 2). cDNA amplification was performed by using the Genesuch DNA amplification reagent kit (Perkin-Elmer-Cetus Instruments). The reaction mixture (100 μl in volume) contained 200 ng of human skin fibroblast cDNAs, 1.0 μM of two primers, 200 μM of each dNTP, and 2.5 unit of Taq DNA polymerase. Fifty cycles of denaturation (94 °C, 60 s), annealing (50 °C, 90 s), and extension (72 °C, 120 s) were carried out in an automatic DNA thermal cycler. Amplified DNAs were separated on a 1.0% agarose gel electrophoresis. The amplified DNA band equivalent to 301 base pairs on agarose gel was excised, electroleutonised, and subsequently subcloned into a SmaI site of M13 mp18 and mp19 sequencing vectors. The correct sequence of PDH Eβ cDNA prepared from human fibroblasts was subsequently confirmed by the DNA sequencing using dideoxynucleotide-chain termination method (23).

RESULTS

Isolation and Characterization of cDNA Clones for Human Pyruvate Dehydrogenase Eβ Subunit—In order to study the structure and regulation of human pyruvate dehydrogenase complex, we isolated cDNA clones for PDH Eβ subunit using an oligodeoxynucleotide probe. Screening more than 400,000 colonies of a human liver 18S rRNA cDNA library, five positive cDNAs with insert sizes ranging from 0.9 to 0.5 kb were identified and plaque purified. All of these were highly homologous to a cDNA clone recently isolated from human foreskin (20). One cDNA was used as a probe to isolate the full-length cDNA clone for PDH Eβ subunit. Finally, two distinct cDNAs with insert sizes of 1.1 and 1.5 kb were isolated, subcloned into plasmid pUC13, and designated pHLPB14 and pHLPB12, respectively, (Fig. 1).

The Nucleotide and Deduced Protein Sequences of Two cDNA Clones for Eβ Subunit—The primary structures of these cDNA clones were determined by nucleotide sequencing using the strategy given in Fig. 1. The sequence of pHLPB14 revealed that it possesses the entire Eβ protein coding region, comprising the leader sequence for the precursor protein and the mature protein coding sequences with the initiation codon ATG and the termination codon TAG. In contrast, pHLPB12 lacks 72 bases of 5'-amino-terminal sequence (Fig. 2). pHLPB14 contained 5 bases of the 5'-untranslated region followed by 1,080 bases of an open reading frame and 44 bases of the 3'-untranslated region including a poly(A) tail which is located 11 bases downstream from the termination codon TAG. The consensus polyadenylation signal AAATAA, 22 bases upstream from the termination codon was not observed in the 3'-untranslated region of pHLPB14, but an alternative one was identified 22 bases upstream to the termination codon TAG. In contrast to pHLPB14, the second cDNA, pHLPB12, had

![Fig. 1. Schematic diagrams of cDNAs encoding for pyruvate dehydrogenase Eβ subunit. The restriction endonuclease maps and sequencing strategies for two types of cDNAs for PDH Eβ subunit, pHLPB14, and pHLPB12 are shown. The solid boxes and the open boxes represent the coding regions and the untranslated regions, respectively. The length and direction of sequenced fragments generated by several restriction endonuclease cleavages are indicated by arrows. Cognate sites on the vertical lines represent the relevant restriction sites of endonucleases: A, AatII; E, EcoRl; P, PstI; Po, PvuII.](image-url)

![Fig. 2. Nucleotide and deduced protein sequences of human liver cDNA clones for pyruvate dehydrogenase Eβ subunit. The nucleotide and deduced protein sequences of the full-length cDNA for PDH Eβ subunit, pHLPB14 is shown. Identical nucleotide sequence of the other cDNA clone, pHLPB12, starting at nucleotide 73, is also designated by the solid lines. Amino acid sequences for the oligodeoxynucleotide probe are denoted by asterisks (*) while the termination codon is shown in the box. Bold underlines represent the potential polyadenylation signal AAATAA, 22 bases upstream from the termination codon in pHLPB14 and 383 bases downstream from the termination codon in pHLPB12. The different amino acid residues between liver and foreskin (20) clones are denoted in the figure.](image-url)
a 400-base 3'-untranslated region including another potential polypadenylation signal AATAAA which was found 383 bases downstream from the termination codon TAG. However, the nucleotide sequence of pHLPB12 for the El β protein coding region is identical with that of pHLPB14. Thus, the deduced protein sequences of pHLPB14 revealed a precursor protein of 359-amino acid residues containing the leader sequence (20) and a mature protein of 329 amino acid residues with molecular weights of 39,223 and 35,894, respectively.

When the deduced protein sequences of our human liver cDNA clones were compared with that of a foreskin cDNA clone (20), three frameshift mutations were detected, one in the leader sequence and two in the protein coding region. As shown in Figs. 2 and 3, the absence of one base (T) between nucleotide position 23-24 and the presence of an additional base (G) at nucleotide position 39 were found in the leader sequence region of the liver cDNA clone, pHLPB14. In the mature protein coding region, the absence of one base at nucleotide positions 635-636 (G) and 928-929 (A) and the presence of an additional base at nucleotide position 638 (C) were identified in both of our liver cDNA clones, pHLPB14 and pHLPB12. Another single base substitution at nucleotide position 438 (A in liver clone, but G in foreskin clone) was found, but it would result in a silent mutation that would not change the amino acid composition. Because of apparent differences in the sequences at nucleotide position 438 (substitution of G to A) and at nucleotide position 935 (addition of T) for the El/3 clones, two additional AauII restriction enzyme sites were generated in the human liver cDNA clones, pHLPB14 and pHLPB12 (Fig. 1), while only one AvaII site was present in the foreskin clone (20). The additional AvaII restriction endonuclease sites were confirmed by the digestion of our cDNA clones with this restriction enzyme (data not shown). Because of the differences in the nucleotide sequences of foreskin and liver, the deduced amino acid sequences for PDH El/3 subunit were different in two clones from two different tissues: 5 residues in the leader region and 11 residues in the mature protein (denoted as bold characters in Fig. 2). The actual autoradiographs of the sequencing gels demonstrated the differences between liver and foreskin cDNA clones in their leader regions (nucleotide position 19-41) and mature protein coding regions (nucleotide position 634-667). Thus, the deduced amino acid sequence for PDH El/3 that we report here might result in alterations in local net charges and protein secondary structures of the leader region (amino acid position 5-15) as well as the mature protein (amino acid positions 208-226 and 304-318) from what was reported by Koike et al. (Table I). In addition to the changes described above, the deduced amino acid sequence in the leader region of liver PDH El/3 contained 3 more arginine residues (5 arginine residues in liver, Fig. 2) than that of foreskin PDH El/3 (20). This finding is of some interest as arginine is thought to play an important role in the protein processing of mitochondrial presequence (32).

In order to verify the differences in nucleotide sequences of the PDH El/3 cDNAs, human skin fibroblast cDNA spanning 301 bases (corresponded to the sequence from nucleotide position 452 to 752 of human liver cDNA in Fig. 1) was amplified by the polymerase chain reaction as described under "Materials and Methods." DNA sequencing (data not shown) of the amplified cDNA revealed identical nucleotide sequence with that of PDH El/3 cDNAs isolated from human liver (Fig. 2). Thus, the differences in the nucleotides and the subsequent translated peptide sequences might be due to cloning artifacts which occurred during the preparation of a foreskin cDNA library or simply misreading of the sequencing data.

Differential Regulation of PDH El, in Various Human Skin Fibroblasts—Recent reports suggest that there are multiple modes of regulation of the PDH El subunit (11, 19, 38-35). To further delineate the biochemical mechanism of PDH-related abnormalities in human subjects, several established

![Fig. 3. The actual autoradiography of the regions demonstrating the differences. The actual autoradiography of sequencing gels demonstrating the differences between liver and foreskin (20) cDNA clones are presented. The regions of nucleotide insertion or deletion in liver cDNAs as compared with foreskin clone are denoted by asterisks (*). Compared with foreskin cDNA, the human liver cDNAs revealed one base T deletion (→ T) and one base G insertion (+G) in leader sequence (left panel), and one base G deletion (→ G) and one base C insertion (+C) in the mature protein coding sequence (right panel). The directions of sequencing gel reading (from 5' to 3' end) are indicated by bold letters with arrows, and numbers in parentheses represent nucleotide position in Fig. 2.](image-url)
fibroblast cell lines from patients with different clinical symptoms (Leigh's syndrome and tricarboxylic acid cycle defective) were selected. The cells were grown in vitro tissue culture and analyzed for the enzyme activity, protein, and mRNA levels. The cell lines used in this study included one control cell line, two from patients with Leigh's syndrome, and one tricarboxylic acid cycle-deficient cell line. The levels of dichloroacetate-stimulated pyruvate dehydrogenase activity in the control fibroblast was 4.0 nmol of acetyl coenzyme A production/min/mg protein. Rather surprisingly, the enzyme activities of whole PDH complex in the fibroblasts from the two Leigh's syndrome patients (GM1503 and GM3672) were 5.5 and 3.0 nmol/min/mg protein, which are comparable to the activity of the control cell. In contrast, in the tricarboxylic acid cycle defective mutant cell, it was about 0.3 nmol/min/mg protein, which is in agreement with an earlier report (11).

The possible mechanism of abnormality in the PDH E1 from these fibroblasts was studied. The amounts of mRNA for PDH E1a and E1b were measured in the various fibroblasts by the Northern blot analyses using PDH E1a and PDH E1b cDNAs as probes. As shown in Fig. 4A, a single species of mRNA (4.8 kb in size) that hybridized with PDH E1a cDNA was observed in all the fibroblasts examined. The amounts of PDH E1a mRNA in the control and patients' fibroblasts were comparable. When another identical gel was subjected to Northern blot hybridization with PDH E1b cDNA, one major species of mRNA (4.4 kb in size) and two minor species (1.8 and 1.1 kb in sizes) were observed (Fig. 4B). The amounts of PDH E1b mRNA in the control and patients' fibroblasts were again almost equal. The similar species and quantities of mRNAs for both PDH E1a and E1b subunits in various cell lines, including the tricarboxylic acid cycle defective mutant, indicated that the defects were not associated with the abnormal expressions of E1a or E1b mRNAs.

The post-transcriptional defect in the tricarboxylic acid cycle defective mutant was further investigated. Immunoblot analyses for PDH E1a and E1b subunits were performed using highly purified polyclonal antibodies against the respective protein subunits of PDH E1a and E1b. Only one immunoreactive PDH E1a band (with an apparent molecular mass of 41,000 daltons) was observed in the whole homogenates from fibroblasts used. The amounts of immunoreactive PDH E1a subunit in fibroblasts from the control and the Leigh's syndrome patients were almost equal and easily detected whereas the amount in the tricarboxylic acid cycle defective mutant was quite low and almost undetectable (Fig. 5A). A similar observation was made with PDH E1b subunit quantified by polyclonal antibody against PDH E1b (Fig. 5B). Only one immunoreactive PDH E1b protein (with an apparent molecular mass of 36,000 daltons) was observed in whole homogenates from all fibroblasts used in this experiment. The amounts of immunoreactive PDH E1b protein in the control and the Leigh's syndrome patients were similar and easily detected while that in the tricarboxylic acid cycle defective mutant was much lower than those of the control cells. The immunoblot data suggested that low enzyme activity observed in tricarboxylic acid cycle defective mutant was due to decreased levels of both PDH E1a and E1b proteins. Similar immunoblot data of decreased levels of PDH E1a and E1b subunits were also observed indicating the same types of defect for these subunits in this particular cell line (data not shown).

Genomic DNA Southern Blot Analysis for PDH E1a Gene

In order to determine whether PDH E1a gene has other closely related gene family members, Southern blot analysis was performed. Total genomic DNAs, isolated from various human skin fibroblasts, were digested with restriction endonucleases, subjected to agarose gel electrophoresis and transferred to GeneScreen membrane. The DNA band hybridization patterns with 32P-labeled E1a cDNA probe were simple and identical for all the genomic DNAs isolated. Only a few fragments were detected in all the cell lines including the control, Leigh's syndrome, and tricarboxylic acid cycle-deficient patient's fibroblasts (Fig. 6). The identical sizes and their simple hybridization pattern with E1b cDNA probe...
The pyruvate dehydrogenase E$_1$ enzyme is a tetramer consisting of two identical E$_{1\alpha}$ and two identical E$_{1\beta}$ subunits. The enzyme is inactivated by phosphorylation and activated by dephosphorylation by a PDH-specific kinase and a PDH-specific phosphatase (2-4), respectively. Its activity is also dependent upon the concentrations of various metabolic regulators such as pyruvate, ATP/ADP, NAD/NADH, and acetyl-coenzyme A/Coenzyme A ratios (36). Although numerous studies were carried out on the function and structure of the E$_{1\alpha}$ subunit (37, 38), relatively little information on the potential role of the E$_{1\beta}$ subunit in PDH activity is available. In this report, we described the isolation and sequence of two distinct cDNA clones for human liver PDH E$_{1\beta}$ which have identical nucleotide sequences for protein coding regions. One clone, pHLBP14, had an unusual polyadenylation signal within the protein coding sequence which is immediately followed by poly(A) tail. Similar unusual cases were recently reported for other cDNA clones for human gonadotropin $\beta$-subunit (39), human factor X (40), and human lecithin-cholesterol acetyltransferase (41). The nucleotide sequence and deduced amino acid sequences in the two cDNAs isolated from human liver revealed that they were highly homologous to those of a foreskin clone (20), except for three regions of frameshift mutations and one base substitution. In this report, we confirmed that our nucleotide sequence of human liver PDH E$_{1\beta}$ cDNA (nucleotide position 452-752 in Fig. 2) was correct. The confirmation was accomplished by the sequencing of the region of difference after skin fibroblast DNA was amplified by the polymerase chain reaction. The exact mechanism for the difference in the nucleotide sequences of PDH E$_{1\beta}$ from human liver and from foreskin (20) is not known, but it could be due to mutations during gene conversions (42), cloning artifacts, or misreading of nucleotide sequences. If the previously predicted structures for foreskin cDNA is incorrect, the frameshifts observed here would result in drastic changes in the local net charges of the amino acids and probably its secondary protein structures. The significance of these alterations in amino acid composition with regard to the changes of catalytic activity awaits further biochemical characterization.

Northern blot analyses of all of the fibroblasts used in our experiments indicated that there are one major species of mRNA for PDH E$_{1\alpha}$ subunit and three species of mRNA for PDH E$_{1\beta}$ subunit. Our data for PDH E$_{1\beta}$ mRNA were in contrast to the results of Koike et al. (20), who reported only one species of mRNA for E$_{1\beta}$ subunit (1.7 kb) in cultured HeLa cells. Although we do not know the reason for the apparent differences, multiple species of E$_{1\beta}$ mRNA were also observed in hamster tissues as well as in rat tissues. The simple patterns of hybridization found in the genomic DNA fragments generated by digestion with different restriction enzymes indicated that PDH E$_{1\beta}$ is not a member of a multigene family. Based on the relatively small size (about 19.7 kb in total size), it is probably derived from a single gene localized on the human chromosome 3 (43). The multiple species of E$_{1\beta}$ cDNA clones and mRNA reported here thus represent the possibility of alternative splicing of a single gene and the usage of different polyadenylation signals during the synthesis and processing of its mRNAs.

In the present study, we also attempted to explore the underlying mechanism of the deficient pyruvate metabolism in some of the well-established fibroblasts from patients who are thought to have defects in PDH E$_1$ subunits. In the two cell lines from Leigh's syndrome patients, we found no abnormality in either PDH E$_{1\alpha}$ or E$_{1\beta}$ subunits as judged by the total enzyme activity, amounts of both mRNA and immunoreactive proteins. The levels of PDH enzyme activities for these fibroblasts appeared to be normal and comparable to those of the control cell lines. The defects in the fibroblasts from Leigh's syndrome patients may not be due to the defects in PDH E$_1$ activity as claimed (10) but rather due to problems of pyruvate transport and uptake through the mitochondrial membrane and defects in other enzyme systems such as PDH phosphatase (44) or cytochrome oxidase (45). Recent reports on the same fibroblasts suggested that the defect is caused by structural abnormality in dihydrolipoamide dehydrogenase (46). The latter claim was based on a relatively lower sensitivity to inhibitory antibodies against lipoamide dehydrogenase and inefficient reconstitution between the subunits to form a catalytically active PDH complex. Our data that failed to demonstrate an abnormality in PDH E$_{1\alpha}$ and E$_{1\beta}$ subunits support their results. On the other hand, in the tricarboxylic acid cycle defective mutant, we observed the reduced levels of immunoreactive PDH E$_{1\alpha}$ and E$_{1\beta}$ subunits with low enzyme activity despite the equivalent amounts of their mRNAs. The results suggested that the defects in this cell line might be due to a post-transcriptional mutation. This may include a defect in translational machinery and inefficient translation of mRNA into subunit proteins. This, in turn, may result in unreliable incorporation of PDH subunits into the mitochondria (47) leading to the rapid degradation of protein despite sufficient levels of protein translation. Alternatively, the
defects.
fects might be due to abnormalities of mitochondrial structures (48) or mitochondrial carrier proteins necessary for efficient pyruvate oxidation (49). Because of the decreased levels of immunoreactive proteins and enzyme activities of all PDH subunits examined, it is more likely that the defect in this cell line could be mainly due to abnormal mitochondrial structures as suggested. However, the exact biochemical mechanisms of the defects in PDH complex enzymes in these established fibroblasts can be further elucidated by the molecular biology techniques such as expression of cloned cDNAs for PDH subunits.

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