0031-3998/91/3001-0011$03.00/0
PEDIATRIC RESEARCH

Vol. 30, No. 1, 1991
Printed in U.S.A.

Demonstration of an Unstable Variant of Pyruvate Dehydrogenase Protein (E_1) in Cultured Fibroblasts from a Patient with Congenital Lactic Acidemia

A. H. M. MAHBUBUL HUQ, MICHINORI ITO, ETSUO NAITO, TAKAHIKO SAIJO, EIJI TAKEDA, AND YASUHIRO KURODA

Department of Pediatrics, School of Medicine, University of Tokushima, Kuramoto Cho 3, Tokushima 770 Japan

ABSTRACT. The deficiency of pyruvate dehydrogenase enzyme complex causes congenital lactic acidemia and devastating neurologic abnormalities in newborns and children. In the majority of cases, the basic defect appears to be in the pyruvate dehydrogenase (E_1) component, which consists of two subunits, α and β. Whereas some patients are deficient of a single subunit, in other patients both subunits of E_1 are missing. To find out why two proteins were deficient, we investigated the cultured fibroblasts of a female patient who had missing E_1-α and E_1-β protein bands on Western blot. Radiolabeling-immunoprecipitation studies with ^{35}S-methionine revealed that patient fibroblasts synthesized normal sized precursor E_1-α and E_1-β proteins, which were presumably transported into mitochondria and processed into normal sized mature proteins. However, pulse-chase analysis showed that α- and β-proteins were degraded rapidly compared to normal. Our findings proved that α- and β-subunits were synthesized and processed normally but failed to form a stable structure for incorporation into the pyruvate dehydrogenase complex. (*Pediatr Res* 30: 11–14, 1991)

Abbreviations

PDHC, pyruvate dehydrogenase complex
E_1, pyruvate dehydrogenase component of PDHC

PDHC, an intramitochondrial multienzyme complex, consists of three catalytic components, two regulatory components, and a component known as Protein-X, whose function is unknown (1). The catalytic components are pyruvate dehydrogenase (EC 1.2.4.1), dihydrolipoyl transacetylase (EC 2.3.1.12), and dihydrolipoamide dehydrogenase (EC 1.6.4.3), commonly referred to as E_1, E_2, and E_3 respectively. The E_1 component is a tetramer of two different subunits, α and β (2). The constituent polypeptides of PDHC, like the majority of mitochondrial proteins, are nuclear coded and translated on cytoplasmic ribosomes as higher molecular weight cytosolic precursors which, after transport into mitochondria, are processed into mature proteins (3).

Defects of PDHC cause congenital lactic acidemia and devastating neurologic abnormalities. Mutation at the active sites of E_1, E_2, E_3, and their cofactor binding sites, and defects of activation of PDHC could lead to PDHC deficiency (4, 5). Deficiencies of nuclear-coded polypeptides may be due to reduced synthesis, increased rate of degradation, or defective transport of protein precursors from cell cytosol to mitochondria. One aspect still awaiting definition is different subunit defects possible in E_1 deficiency (6). There is heterogeneity in the expression of E_1 deficiency at the protein level. Examination of deficient cell lines at the level of mRNA by Northern blotting techniques indicated that, although in some patients the defect was in the synthesis of E_1 protein, in other patients the defect could be due to some other mechanisms (7). In some patients, both E_1-α and E_1-β were deficient (7–9). To find the mechanism of combined deficiency, we investigated the synthesis, mitochondrial import, processing, and rate of degradation of both proteins in cultured fibroblasts of a patient with PDHC deficiency.

MATERIALS AND METHODS

Brief case report. The patient was a girl born at the 38th wk of an uncomplicated pregnancy. The birth weight was 2590 g. Neither of the parents had any problem. The patient presented with poor feeding, muscle weakness, developmental retardation, and lactic acidosis. Serum lactic and pyruvic acid levels were 4.57 mmol/L (41.2 mg/dL) and 0.44 mmol/L (3.89 mg/dL), respectively. Urine organic acids and plasma amino acids were uninformative. At 8 mo of age, she developed infantile spasm. Computed tomography scan showed total brain atrophy. EEG showed diffuse spikes and waves. At 4 y 9 mo of age, enzyme studies on a biopsy specimen of muscle and cultured fibroblasts showed PDHC deficiency (10). The original and dichloroacetic acid-activated activities were 20 and 9% of normal, respectively. Total activity of PDHC, measured after *in vitro* activation with an excess amount of a broad specificity phosphatase, was 16% of normal. The patient was maintained on a respirator from 6 y of age until her death at the age of 8 y.

Materials. L-^{35}S-methionine (>800 Ci/mmol) and ^{14}C-labeled protein standard were purchased from Amersham Corp. (Arlington Heights, IL). Formalin-fixed *Staphylococcus aureus* cells were from Bethesda Research Laboratories (Bethesda, MD). Rhodamine 6G, x-ray film, X-Omat R, and film cassettes were from Eastman Kodak Co. (Rochester, NY). The immunoblot system was purchased from Bio-Rad Laboratories (Richmond, CA). Cycloheximide, phenylmethanesulphonyl fluoride, *p*-aminobenzamidine, and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO). Cell culture materials were from Nissui, Tokyo, Japan.

Preparation of antibodies against PDHC and E_1-α and E_1-β proteins. The PDHC was purified from ox heart by the method of Stanley and Perham (11). On SDS-PAGE, PDHC gave the

Received January 4, 1991; accepted March 4, 1991.
Correspondence: A.H.M. Mahbubul Huq, M.B.B.S., Ph.D., Department of Pediatrics, School of Medicine, University of Tokushima, Kuramoto Cho 3, Tokushima 770, Japan.
Supported by grants from the National Center of Neurology and Psychiatry (NCNP) of the Ministry of Health and Welfare (6-A) and from the Ministry of Science, Education and Culture, Japan (63570441).

following bands: E_1-β, E_1-α, E_2, E_3, and X. The different components were separated by SDS-PAGE and capillary endomosis using the electro-elutor ELFE as described in the manual (Genofit, Switzerland). Rabbit antibodies were prepared against PDHC E_1-α and E_1-β proteins using standard procedures.

Immunoblot analysis. The confluent monolayers of cultured skin fibroblasts were washed with ice-cold PBS and suspended in a solution of 20 mM potassium phosphate, pH 7.0, 0.25 M sucrose, 1 mM EDTA, and 5 mg/L of leupeptin. The cells were treated with digitonin (1 mg/mL), and the mitochondrial fraction was collected by centrifugation. The pellet was resuspended in a solution of 20 mM potassium phosphate, pH 7.0, 1% Triton X-100, 3 mM EDTA, 0.5 mg/L of leupeptin, and 0.2 mM phenylmethylsulphonyl fluoride and freeze-thawed three times at −80°C. The soluble fraction was stored in aliquots of 50 μg of protein at −80°C until use. SDS-PAGE was done with 10% separating gel and 4% stacking gel as described by Laemmli (12). After separation by SDS-PAGE, proteins were transblotted to nitrocellulose membranes as described in the instruction manual. The blots were blocked in Tris-buffered saline containing 3% nonfat dry milk and incubated for 1 h in anti-PDHC antibody diluted 1:500 with Tris-buffered saline. Components were detected by a Bio-Rad antirabbit immune-blot horseradish peroxidase kit.

Pulse-labeling of fibroblasts with ^{35}S-methionine and pulse-chase. Confluent monolayers of fibroblasts in a 25-cm^2 flask were labeled with ^{35}S-methionine (50 μCi) in 2.5 mL of labeling medium containing 60% Puck's saline G, 15% dialyzed FCS, and 10% glucose (12). Optimum labeling for E_1-β and E_1-α occurred when the culture flasks were incubated 1 h and 4 h, respectively. After incubation, the labeling medium was removed and cells were washed twice with PBS and then harvested by adding 5 mL of NETS/methionine buffer (150 mM NaCl, 10 mM EDTA, pH 7.4, 0.5% Triton X-100, 0.25% SDS, and 2% unlabeled methionine) to each flask. When studying the precursor proteins, labeling was done in the presence of 1 μm of rhodamine 6G. In the chase experiments, after pulse-labeling, the medium was replaced with growth medium containing no label, and the cells were harvested at 24, 48, 72 and 96 h, respectively. Optimal conditions for immunoprecipitation of PDHC or individual subunits were determined in pilot studies. ^{35}S-methionine incorporation into total proteins was measured by a liquid scintillation counter (Aloka 612, Tokyo, Japan) and the amount containing 10^7 dpm was used for immunoprecipitation. The individual subunits were immunoprecipitated with anti-E_1-α and anti-E_1-β antibody and *S. aureus* cells. The precipitates were analyzed by SDS-PAGE and fluorography. Protease inhibitors were present in all subsequent steps during the immunoprecipitation procedure.

RESULTS

Immunochemical analysis. Figure 1 shows the immunoblot of SDS-PAGE of purified bovine PDHC (*lane 1*) and mitochondrial extract from normal (*lanes 2* and *4*) and PDHC-deficient (*lane 3*) fibroblasts. Mitochondrial extract of normal fibroblasts gave four bands corresponding to components E2, X, E_1-α and E_1-β. Patient fibroblasts gave normal E2 and X bands, but E_1-α and E_1-β bands were missing. The E3 band could not be detected by Western blot analysis in either patient or in normal fibroblasts because of its low immunogenecity. Western blot analysis using monospecific antibodies against E_1-α and E_1-β confirmed absence of E_1-α and E_1-β bands in patient fibroblasts (result not shown).

Electrophoretic analysis of E_1-α and E_1-β proteins labeled with ^{35}S-methionine. We studied the synthesis and mitochondrial import of the precursors of α- and β-subunits of E_1 protein in normal and patient fibroblasts by labeling with ^{35}S-methionine in presence of rhodamine 6G. Rhodamine 6G, an inhibitor of the mitochondrial energy metabolism, is known to inhibit the uptake of precursor of nuclear-encoded mitochondrial enzymes,

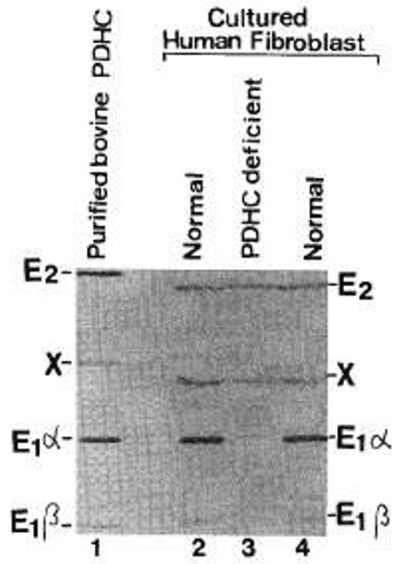

Fig. 1. Immunoblot of PDHC in extracts of normal and PDHC-deficient cultured skin fibroblast mitochondria. Extracts of cultured skin fibroblast mitochondria (50 μg protein/sample) were separated by SDS-PAGE and electroblotted to a filter membrane. PDHC components were detected with anti-PDHC antibodies and a Bio-Rad antirabbit immunoblot horseradish peroxidase kit. *Lane 1*, purified ox heart PDHC (300 ng protein). *Lanes 2* and *4*, mitochondrial extract from normal fibroblasts. *Lane 3*, mitochondrial extract from PDHC-deficient fibroblasts.

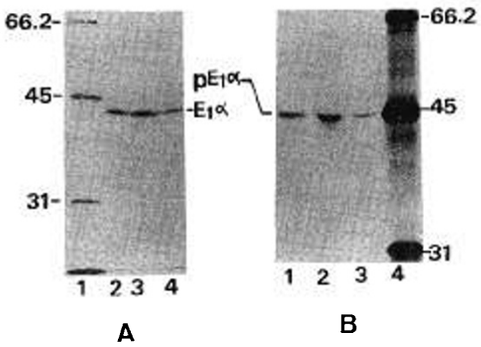

Fig. 2. Detection of precursor ($pE_1\alpha$) and mature ($E_1\alpha$) E_1-α protein in normal and PDHC-deficient fibroblast. The confluent monolayers of fibroblasts were labeled with ^{35}S-methionine for 4 h in the presence (*B*) and in the absence (*A*) of rhodamine 6G, an inhibitor of mitochondrial import. After harvesting with NETS/methionine buffer, monospecific anti-E_1-α antibody and *Staphylococcus* cells were added to the fibroblast extracts and the immunoprecipitates (total radioactivity into incorporated protein, 10^7 dpm) were analyzed by 10% SDS-PAGE and fluorography. *A*, *lanes 2* and *4*, mature E_1-α protein from normal fibroblasts. *A*, *lane 3*, mature E_1-α protein from patient fibroblasts. *B*, *lanes 1* and *3*, precursor E_1-α protein from normal fibroblasts. *B*, *lane 2*, precursor E_1-α protein from patient fibroblasts. *Lane 1* in *A* and *lane 4* in *B* were standard molecular weight markers.

leading to the suppression of their posttranslational processing (13–15). In normal fibroblasts, when E_1-α protein was labeled in absence of rhodamine 6G, a sharp band with a molecular weight of 42 kD was observed (Fig. 2*A*, *lanes 2* and *4*). In patient fibroblasts, the band had the same molecular weight but was consistently more intense (Fig. 2*A*, *lane 3*). When cell labeling was performed in the presence of rhodamine 6G, the 42-kD band was not present and a new 44.5-kD band for precursor protein was detected in both normal and patient fibroblasts (Fig. 2*B*, *lanes 1*, *2*, and *3*). In Figures 2*A* and *B*, the bands in the patient's lanes were more intense than those of the corresponding control. This might reflect increased synthesis or altered immu-

noreactivity of a variant E_1-α protein. Both 42- and 44.5-kD bands were not detectable when an excessive amount of pure E_1-α was added to each immunoprecipitation mixture before the addition of antibody (result not shown). This confirms that 42- and 44.5-kD bands were indeed the mature and precursor E_1-α, respectively.

Using anti-E_1-β antibody, a 36-kD band was detected in both normal and patient fibroblasts (Fig. 3, *lanes 1* and *3*) when labeling was done in the absence of rhodamine 6G. When labeling was done in the presence of rhodamine 6G, the 36-kD band was not present, and a new 39-kD band for precursor protein was detected in both normal and patient fibroblasts (Fig. 3, *lanes 2* and *4*). Both 36- and 39-kD bands disappeared when excess E_1-β protein was added to each immunoprecipitation mixture before the addition of antibody (result not shown).

Pulse-labeling chase experiment. To investigate the rate of degradation, we pulse-labeled E_1-α protein for 4 h with ^{35}S-methionine and then chased the labeled E_1-α for various periods. The density of the fluorographic band for E_1-α remained the same at 24 h of chase in the normal fibroblast line (Fig. 4, *lane 2*) but decreased markedly in the patient fibroblasts (Fig. 4, *lane 5*). The E_1-α protein band in patient fibroblasts almost disappeared after 48 h, although the band in the normal fibroblasts did not change much. In the normal fibroblasts, the fluorographic band was clearly detectable even after 10 d (result not shown).

Similarly, we did a pulse-chase experiment for E_1-β protein (Fig. 5). The density of the fluorographic band for E_1-β protein remained comparable at 24 h (result not shown) and decreased progressively at 48 and 96 h, but was clearly detectable at 96 h in the normal fibroblast line (Fig. 5*A*, *lanes 1–3*). In the normal fibroblast line, the fluorographic band was detectable even after 10 d (result not shown). The patient fibroblast line, after 1 h of pulse-labeling, synthesized E_1-β protein in an amount comparable to the normal fibroblast line (Fig. 5*B*, *lane 1*). However, the fluorographic band was barely detectable after 48 h of chase (Fig. 5*B*, *lane 2*) and could not be detected after 96 h of chase (Fig. 5*B*, *lane 3*), even when the film was exposed for a long time.

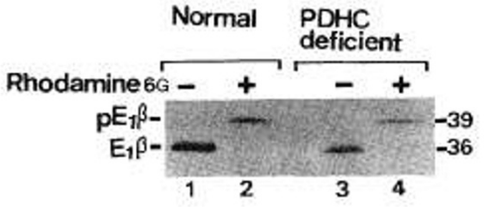

Fig. 3. Detection of mature ($E_1\beta$) and precursor ($pE_1\beta$) E_1-β protein. After labeling for 1 h with ^{35}S-methionine, E_1-β was immunoprecipitated and analyzed by SDS-PAGE and fluorography. *Lane 1*, normal mature protein. *Lane 2*, normal precursor protein. *Lane 3*, patient mature protein. *Lane 4*, patient precursor protein.

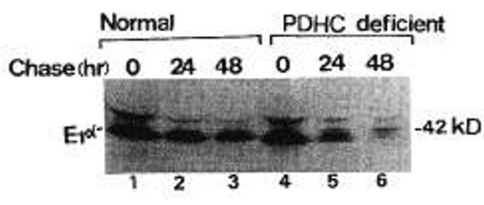

Fig. 4. Pulse-labeling chase experiment for E_1-α protein in normal and PDHC-deficient fibroblasts. Confluent monolayers of fibroblasts, in 25-cm^2 flasks were labeled with ^{35}S-methionine. After 4 h of labeling, the labeling medium was replaced with normal growth medium and the preparation chased for 24 and 48 h. After chase, labeled E_1-α protein was immunoprecipitated and analyzed by 10% SDS-PAGE and fluorography. *Lanes 1–3*, normal fibroblasts. *Lanes 4–6*, patient fibroblasts. *Lane 1* and *4*, immediately after 4 h of pulse. *Lanes 2* and *5*, after 24 h of chase. *Lanes 3* and *6*, after 48 h of chase.

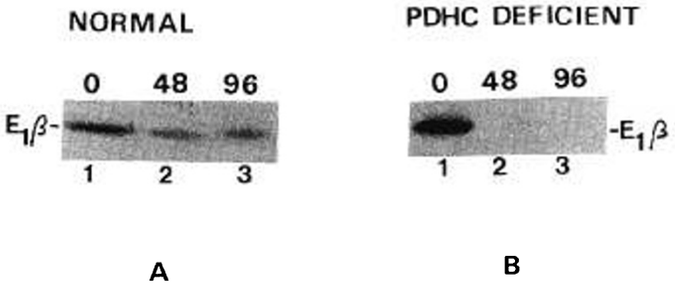

Fig. 5. Pulse-chase experiment for E_1-β protein in normal and PDHC-deficient fibroblasts. After labeling for 1 h with ^{35}S-methionine, the labeling medium was replaced with normal growth medium and the preparation chased for 48 and 96 h. *A*, *lanes 1–3*, normal. *B*, *lanes 1–3*, patient. *A* and *B*, *lane 1*, immediately after 1 h of pulse. *Lane 2*, after 48 h of chase. *Lane 3*, after 96 h of chase.

DISCUSSION

We investigated cultured fibroblasts from a patient with PDHC deficiency. Immunoblot and radiolabeling-pulse-chase experiments showed increased degradation of E_1-α and E_1-β subunits.

Deficiency of PDHC is the most common of the disorders leading to congenital lactic acidemia (6). More than 100 cases of this deficiency have been described in the literature (16). In the majority of cases, the basic defect appears to be in the E_1 protein, and different patterns of E_1 deficiency have been detected (6–9). In one group, there are immunologically detectable E_1-α and E_1-β with normal mRNA levels. A second group has diminished amounts of both immunoreactive proteins, but normal levels of mRNA for both subunits. In another group, immunologically detectable E_1-α and E_1-β proteins are diminished, but with reduced levels of mRNA for E_1-α only. In a fourth group there are few cases of isolated E_1-α deficiency. It is striking that only E_1-α or combined deficiency have been reported. The phosphorylation sites for regulation of the whole complex are on the E_1-α subunit; it may be that a defect involving the E_1-β chain would not have the same severe consequences as a lack of or a disturbance of phosphorylation of α-subunit.

It is interesting that both E_1-α and E_1-β proteins were deficient in our patient and in some reported cases (7, 8). E_1-α and E_1-β proteins are encoded by different genes (17). This excludes the possibility that a common mRNA and protein exist from which E_1-α and E_1-β are derived. The chances that there were simultaneous mutations in two different genes encoding both the subunits in a group of unrelated patients are very small. However, there are instances in biology where a single gene defect causes multiple missing proteins (18). A single gene defect that results in deficiencies of both subunits of the E_1 protein could have several explanations: *1*) mutation in a regulatory gene that controls the synthesis of α- and β-subunits; *2*) a common defect in mitochondrial import and processing of both proteins; and *3*) failure to form a stable $\alpha_2\beta_2$-tetramer for incorporation into PDHC resulting in the degradation of unassociated subunit proteins. A similar case is found in cAMP-dependent protein kinase, where the degradation of the regulatory subunit I is increased 10 times in absence of the catalytic subunit (19).

In this study, immunoblot analysis of PDHC revealed that immuno-cross-reactive E_1-α and E_1-β were absent, indicating that the steady state amount of α- and β-subunits within fibroblasts were very low. However, radiolabeling studies revealed that the same fibroblast line could produce ^{35}S-methionine-labeled E_1-α and E_1-β in amounts comparable to those of normal fibroblast lines. This indicated that patient fibroblasts produced E_1-α and E_1-β proteins that were extremely labile compared to normal proteins. Pulse-chase experiments also confirmed that α- and β-proteins were degraded more rapidly in patient fibroblasts than in normal fibroblasts. The half-life of the precursor protein in cytosol may be shorter than that of the mature form in the mitochondria. However, in patient fibroblasts, E_1-α and E_1-β

proteins were synthesized as 44.5- and 39-kD precursors, which were presumably imported into the mitochondria and processed to 42- and 36-kD mature forms, respectively, in a normal fashion. This excludes the possibility that patient fibroblasts produced truncated precursors that could not properly be imported into mitochondria. Thus, it seems that patient fibroblasts expressed and processed α- and β-subunits normally but failed to form a stable structure for incorporation into the complex; as a result, both the subunits were rapidly degraded. Incidentally, members of the heat shock protein family, a class of well-documented and highly conserved polypeptides, act as chaperons in the folding and assembly of imported subunits (20). Disturbance of a heat shock protein may be important in selected cases of PDHC deficiency. Recently, we have located a 4-bp insertion in the cDNA for E_1-α that resulted in an early stop codon and a shorter E_1 protein (Ito M, Saijo T, Naito E, Takeda E, Huq AHMM, Kuroda Y, unpublished data). The mutant E_1-α protein might affect the proper folding of E_1-α protein and formation of a stable $\alpha_2\beta_2$-tetramer to be incorporated into highly structured PDHC. Mammalian PDHC is composed of a structural core of E_2 protein around which E_1 and E_3 are arranged (1). In our case and also in the earlier reports (7–9), defects in the E_1 component didn't cause any abnormality of E_2 or X in immunoblot analysis. However, in a case of apparent X component deficiency, E_1-α and E_1-β were reduced below normal (21). Further studies of the nature of the possible interactions of different subunits, their states of aggregation, and their assembly are necessary to address the question how a single mutation would affect the assembly and function of PDHC.

REFERENCES

1. Yeamen SJ 1989 The 2-oxo acid dehydrogenase complex: recent advances. Biochem J 257:625–632
2. Reed LJ 1981 Regulation of mammalian pyruvate dehydrogenase complex by a phosphorylation-dephosphorylation cycle. Curr Top Cell Regul 18:95–106
3. Olga GL, Marcucci D, Gibb GM, Dick J, Lindsay JG 1988 Biosynthesis, import and processing of precursor polypeptides of mammalian mitochondrial pyruvate dehydrogenase complex. Biochem J 251:817–823
4. Stansbie D, Wallace SJ, Marsac C 1986 Disorders of the pyruvate dehydrogenase complex. J Inherited Metab Dis 9:105–119
5. Toshima K, Kuroda Y, Takeda E, Watanabe T, Ito M, Naito E, Miyao M 1984 *In vitro* activation of the pyruvate dehydrogenase complex in human muscle by a broad specificity protein phosphatase. J Inherited Metab Dis 7:143–144
6. Robinson BH 1989 Lactic acidemia. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The Metabolic Basis of Inherited Disease. McGraw-Hill Inc, New York, pp 869–888
7. Wexler ID, Kerr DS, Ho L, Pepin MM, Javed RA, Mole JE, Jesse BW, Thekkumkara TJ, Pons G, Patel MS 1988 Heterogenous expression of protein and mRNA in pyruvate dehydrogenase deficiency. Proc Natl Acad Sci USA 85:7336–7340
8. Ho L, Hu CWC, Packman S, Patel MS 1986 Deficiency of the pyruvate dehydrogenase component in pyruvate dehydrogenase complex deficient human fibroblasts. Immunological identification. J Clin Invest 78:844–847
9. Old SE, DeVivo DC 1989 Pyruvate dehydrogenase complex deficiency: biochemical and immunoblot analysis of cultured skin fibroblasts. Ann Neurol 26:746–751
10. Naito E, Kuroda Y, Takeda E, Yokota I, Kobashi H, Miyao M 1988 Detection of pyruvate metabolism disorders by culture of skin fibroblasts with dichloroacetate. Pediatr Res 23:561–564
11. Stanley CJ, Perham RN 1980 Purification of 2-oxo acid dehydrogenase multienzyme complexes from ox heart by a new method. Biochem J 191:147–154
12. Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
13. Ikeda Y, Keese SM, Fenton WA, Tanaka K 1985 Molecular heterogeneity of variant isovaleryl-CoA dehydrogenase from cultured isovalerimic acidemia fibroblasts. Proc Natl Acad Sci USA 82:7081–7085
14. Morita T, Mori M, Ikeda F, Tatibana M 1982 Transport of carbamyl phosphate synthetase I and ornithine transcarbamylase into mitochondria. Inhibition by rhodamine 124 and accumulation of precursors in isolated hepatocytes. J Biol Chem 257:10547–10550
15. Fenton WA, Hack AM, Kraus JP, Rosenberg LE 1987 Immunochemical studies of fibroblasts from patients with methylmalonyl-Coa mutase apoenzyme deficiency: detection of a mutation interfering with mitochondrial import. Proc Natl Acad Sci USA 84:1421–1424
16. Robinson BH, Macmillan H, Petrova-Benedict R, Sherwood WG 1987 Variable clinical presentation in patients with deficiency of the pyruvate dehydrogenase complex. A review of 30 cases with a defect in the E_1 component of the complex. J Pediatr 111:525–533
17. Koike K, Ohta S, Urata Y, Kagawa Y, Koike M 1988 Cloning and sequencing of cDNAs encoding α and β subunits of human pyruvate dehydrogenase. Proc Natl Acad Sci USA 85:41–45
18. Luck DJL 1984 Genetic and biochemical dissection of the eucaryotic flagellum. J Cell Biol 98:789–794
19. Uhler MD, McKnight GS 1987 Expression of cDNAs for two isoforms of the catalytic subunit of cAMP-dependent protein kinase. J Biol Chem 262:15202–15207
20. Rothman JE, Kornberg RD 1986 An unfolding story of protein translocation. Nature 322:209–210
21. Robinson BH, Mackay N, Petrova-Benedict R, Ozalp I, Coskun T, Stacpoole PW 1990 Defects in the E2 lipoyl transacetylase and the X-lipoyl containing component of the pyruvate dehydrogenase complex in patients with lactic acidemia. J Clin Invest 85:1821–1824