Glutaric Acidemia Type II

Heterogeneity in β-Oxidation Flux, Polypeptide Synthesis, and Complementary DNA Mutations in the α Subunit of Electron Transfer Flavoprotein in Eight Patients

Eric Freneaux, Val C. Sheffield, Lisa Molin, Ann Shires, and William J. Rhead
Department of Pediatrics, University of Iowa, Iowa City, Iowa 52242

Abstract

We studied metabolic, polypeptide and genetic variation in eight glutaric acidemia type II (GA II) patients with electron transfer flavoprotein (ETF) deficiency. As measured by ^{3}H-fatty acid oxidations in fibroblasts, β-oxidation pathway flux correlated well with clinical phenotypes. In six patients with severe neonatal onset GA II, oxidation of [9,10(n)-^{3}H]-palmitate ranged from 2% to 22% of control and of [9,10(n)-^{3}H]myristate, from 2% to 26% of control. Of two patients with late onset GA II, one had intermediate residual activities with these substrates and the other normal activities. Radiolabeling and immunoprecipitation studies revealed that three of the six neonatal onset GA II patients had greatly diminished or absent α- and β-ETF subunits, consistent with a failure to assemble a stable heterodimer. Another neonatal onset patient showed normal synthesis of β-ETF but decreased synthesis of α-ETF. Two neonatal onset and two late onset GA II patients showed normal synthesis of both subunits. Analysis of the pre-α-ETF coding sequence revealed seven different mutations in the six patients with neonatal onset GA II. The most common mutation was a methionine for threonine substitution at codon 266 found in four unrelated patients, while all the other mutations were seen in single patients. No mutations were detected in the two patients with late onset GA II. (*J. Clin. Invest.* 1992. 90:1679–1686.) Key words: denaturing gradient gel electrophoresis • electron transfer flavoprotein • fatty acid oxidation • mitochondria • polymerase chain reaction

Introduction

Glutaric acidemia type II (GA II)[1] is an inborn error of fatty acid and amino acid metabolism characterized by hypoketotic hypoglycemia, metabolic acidosis, and a characteristic organic aciduria. The disease is due to a defect in either electron transfer flavoprotein (ETF) or ETF-ubiquinone oxidoreductase (ETF-QO), two mitochondrial enzymes required for the transfer of electrons between several mitochondrial matrix dehydrogenases and the main electron transport chain (1). Both deficiencies are inherited as autosomal recessive traits (2). ETF is a heterodimer consisting of an α and a β subunit (α-ETF and β-ETF), both nuclear coded and synthesized in the cytosol. α-ETF, but not β-ETF, is synthesized as a precursor (pre-α-ETF) larger than the mature form (3). The pre-α-ETF cDNA coding region comprises 999 base pairs encoding 333 amino acids, with molecular masses of precursor and mature α-ETF estimated to be 35 and 32 kD, respectively (4). Although clinical and enzymatic heterogeneity has been well documented in ETF-deficient GA II patients (2, 5), the molecular basis of this defect is less well understood, with only one patient reported as having a glycine to valine substitution at codon 157 (Val157Gly) in the sequence of pre-α-ETF (6).

In this study, we have examined the biochemical and polypeptide phenotypes and the molecular defects in eight ETF-deficient GA II patients. We measured the oxidation of [9,10(n)-^{3}H]palmitate and -myristate to quantitate β-oxidation flux in GA II cells. We investigated the biosynthesis of both ETF subunits in fibroblasts from these eight patients by radiolabeling and immunoprecipitation with anti-ETF antiserum. Furthermore, we have studied pre-α-ETF cDNA using GC-clamped denaturing gradient gel electrophoresis (DGGE; 7–9), single-stranded conformation polymorphisms (SSCP), direct DNA sequencing, and restriction endonuclease analyses to characterize the molecular defects in these patients.

Methods

Fibroblast and cell culture. Fibroblasts were grown in Eagle's minimal essential medium with Earle's balanced salts, L-glutamine, and nonessential amino acids (JRH Bioscience, Lenexa, KS) containing 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) and antibiotics (penicillin, streptomycin, and gentamycin, Sigma Chemical Co., St. Louis, MO). Control cultures were established from the foreskins of three healthy male infants and from skin biopsies of seven females and three males in whom the diagnosis of GA II was ruled out on the basis of clinical and biochemical analyses and tritiated palmitate assay results. The ETF-deficient GA II patients 1430 (10), 1728 (11), 1803, 1863, 1903, 1916, and 1966 (2) have been described previously. Patient 1430 was identified by Indo et al. (6) as patient YH1313 and by Loehr et al. (2) as patient 1902. Patient 9001 developed hypoglycemia, hyperammonemia, and metabolic acidosis soon after birth. The diagnosis of GA II was made after identification of characteristic plasma acylcarnitine and urine organic acid profiles (personal communication, Dr. G. J. Mick, University of Rochester).

Materials. Chemicals purchased were of the highest grade available. Tran ^{35}S-label (1,000 Ci/mmol, 10 mCi/ml) was purchased from ICN Biomedicals (Costa Mesa, CA). Fixed protein A-positive *Staphylococcus aureus* cells and deoxynucleotide triphosphates were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Entensify was from DuPont-New England Nuclear (Wilmington, DE). Antisera against pig ETF were raised in New Zealand white rabbits as de-

Address reprint requests to Dr. Rhead, Division of Medical Genetics, Department of Pediatrics, University of Iowa, Iowa City 52242.

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1. *Abbreviations used in this paper:* DGGE, denaturing gradient gel electrophoresis; ETF, electron transfer flavoprotein; GA II, glutaric acidemia type II; PCR, polymerase chain reaction; QO, ubiquinone oxidoreductase; SSCP, single-stranded conformation polymorphism(s).

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scribed earlier for our antisera against short- and medium-chain acyl-coenzyme A dehydrogenases (12). Pure ETF was kindly provided by Dr. F. Frerman, University of Colorado, Denver, CO, and Dr. C. Thorpe, University of Delaware, Newark, DE. Taq DNA polymerase and PCR buffer were from Perkin Elmer Cetus (Norwalk, CT). The oligonucleotide primers were synthesized on a model 391 DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). [9,10(n)-^{3}H]-myristic and -palmitic acids and deoxyadenosine 5'-α-[^{35}S] thiotriphosphate, triethylammonium salt (1,000 Ci/mmol, 10 mCi/ml) were purchased from Amersham Corp. (Arlington Heights, IL). Random hexamer was from Pharmacia, Inc. (Piscataway, NJ). Mo-MLV reverse transcriptase was from Bethesda Research Laboratories (Gaithersburg, MD). RNAsin was from Promega Corp. (Madison, WI). Fok I and Nla IV were purchased from New England Biolabs (Beverly, MA).

Tritiated palmitate and myristate oxidations. Oxidation of [9,10(n)-^{3}H]palmitate (20 μM) and -myristate (80 μM) to ^3H_2O in fibroblast monolayers was quantitated as described earlier (5, 13).

Immunoprecipitation of radiolabeled ETF subunits. Immunoprecipitation with rabbit anti-ETF antiserum was carried out according to Ikeda et al. (14), except that the cells were incubated with labeling medium for 12 h instead of 1 h and that two of the four *S. aureus* pellet washes with NaCl, Tris, Triton, deoxycholate, and SDS, and the two H_2O washes were omitted. The immunoprecipitated samples were analyzed by SDS-PAGE using 10% gels as described by Laemmli (15). The gels were treated for fluorography with Entensify and exposed onto X-OMAT AR film (Eastman Kodak Co., Rochester, NY) at −80°C.

RNA preparation. RNA was extracted from cultured fibroblasts according to the method of Chomczynski and Sacchi (15), with the omission of the reprecipitation step and the addition of a second wash with 75% ethanol. The final RNA pellet was resuspended in diethyl pyrocarbonate-treated water and kept at −80°C until used.

cDNA synthesis. Approximately 1 μg of total RNA was mixed in a 20-μl reaction volume containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 1 mM of each deoxynucleotide triphosphate, 2.5 μM random hexamer, 20 U of RNAsin and 50 U of Mo-MLV reverse transcriptase. The reverse transcription reaction was performed using a DNA thermal cycler (Perkin Elmer Cetus) as follows: 10 min at 23°C, 45 min at 37°C, 5 min at 99°C, and 5 min at 5°C.

DNA amplification. The entire coding region of the gene was amplified in five overlapping segments. The primer sequences were designed from the published sequence of pre-α-ETF cDNA (4). For each pair of primers, a 39-bp GC-rich sequence was added at the 5' end of one of the primers to allow optimum analysis by DGGE (9). To the 20-μl reverse transcriptase reaction mixture were added 80 μl of a solution containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.25 mM $MgCl_2$, 0.5 μM of each appropriate primer and 2 U of Taq DNA polymerase. The samples were overlaid with 70 μl of mineral oil. The amplification reaction was performed for 35 cycles in a thermal cycler using the following cycle conditions: 30-s denaturation at 94°C, 30-s annealing at 55°C, and 1-min extension at 72°C. The final extension was for 5 min. A 10-μl aliquot of the amplification product was electrophoresed on a 1.5% agarose gel to confirm successful amplification.

DGGE. The optimum gradient condition and length of electrophoresis were determined for each amplified DNA segment by performing perpendicular DGGE and running travel schedule gels as described elsewhere (17). 17 μl of amplification product was mixed with 5 μl of loading buffer (30% glycerol, 10 mM Tris-HCl, pH 7.8, 1 mM EDTA and 0.1% orange G), and loaded on a 7% (14% for segment 1) polyacrylamide gel (37.5:1, acrylamide:*N,N'*-methylene bisacrylamide) containing the appropriate linear gradient of DNA denaturant (Table I). Electrophoresis was carried out at 60°C at 150-V constant voltage for the appropriate length of time (Table I), as described. Gels were stained with ethidium bromide.

Purification of PCR products for DNA sequencing. 200 μl of the amplification product was electrophoresed on a 1% agarose gel. The DNA fragment was recovered by cutting the desired band from the agarose gel, freezing at −20°C overnight, and centrifugation in a Costar spin-X0.22 μm cellulose acetate filter unit. The eluate was mixed with 1 μl of glycogen (20 mg/ml), 0.2 vol of 5M ammonium acetate, and 2.5 vol of isopropanol and left 30 min on ice. The precipitated DNA was pelleted by centrifugation, washed twice with 70% ethanol, dried, and resuspended in 30 μl of water. In order to sequence independently two different alleles from a single individual, some samples were electrophoresed on a denaturing gradient gel containing a narrow denaturant range (10%) centered on the empirical melting point of the DNA fragments. The segments of the gel containing the DNA fragment corresponding to each allele were excised, soaked in water for 15 min, and incubated overnight with 2 vol of 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8.0, and 0.1% SDS at 37°C with shaking (18). The supernatant was centrifuged in a Costar spin-X 0.22 μm cellulose acetate filter unit to remove residual gel fragments. DNA was precipitated with ethanol, washed with 70% ethanol, dried, and resuspended in water. The DNA resulting from a 350-μl polymerase chain reaction (PCR) reaction was resuspended in 15 μl of water.

Direct sequencing of double-stranded DNA. The sequencing reaction based on the dideoxy termination method of Sanger et al. (19) was performed using Sequenase 2.0 sequencing kit (United States Biochem-

Table I. Optimal Conditions for DGGE of DNA Segments Derived from Pre-α-ETF

DNA segment	Primer characteristics*	Size of amplified product	Denaturing gradient‡	Electrophoresis time
		bp	%	*h*
1	1.1 sense 5' A(−26) → (−1)C 3'			
	1.2 antisense GC-clamp 5' A171 → 151G 3'	237	45–65	10
2	2.1 sense 5' C120 → 140T 3'			
	2.2 antisense GC-clamp 5' A383 → 364A 3'	303	40–65	6
3	3.1 sense GC-clamp 5' G334 → 356T 3'			
	3.2 antisense 5' A676 → 654G 3'	382	35–65	7
4	4.1 sense GC-clamp 5' T621 → 642T 3'			
	4.2 antisense 5' C830 → 808G 3'	248	40–65	7
5	5.1 sense GC-clamp 5' A775 → 795G 3'			
	5.2 antisense 5' T1061 → 1032T 3'	326	20–45	7

* GC-clamp = 5' CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCC 3'.
The numbers indicate the position of the first and last nucleotides of the primer within pre-α-ETF sequence as designated in Finocchiaro et al. (4).
‡ 100% denaturant = 7 M urea and 40% formamide.

ical, Cleveland, OH) with a modified protocol. Bidirectional sequencing was performed using the same primers used for the amplification reactions. A 10-μl reaction containing 7 μl of purified DNA, 2 μl of reaction buffer, and 25 pmol of the appropriate primer was incubated at 95°C for 4 min and immediately transferred to a dry ice-ethanol bath for 1 min. Immediately after thawing, a mixture containing 1 μl of Mn buffer, 1 μl of [^{35}S]dATP, 2 μl of a 1:20 dilution of labeling mix, 1 μl of dithiothreitol, and 2 μl of a 1:8 dilution of Sequenase enzyme was added. The labeling reaction was performed for 8 min at room temperature. Termination was performed for 5 min as described by the manufacturer except that it was performed at 40°C. Sequencing reactions were analyzed on 6% polyacrylamide denaturing gels. Gels were transferred onto Whatman Inc. (Clifton, NJ) 3MM paper, dried, and autoradiographed with Kodak X-OMAT AR film.

Identification of SSCP. DNA amplification was performed as described above, except that the reaction volume was scaled down to 10 μl and contained 4 μCi of [^{35}S]dATP. 1 vol of PCR product was mixed with 3 vol of a solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol. 5 μl of this mixture was heated at 95°C for 4 min and loaded onto 6% nondenaturing polyacrylamide gel (49:1, acrylamide:*N,N'*-methylene bisacrylamide) containing 5% glycerol in 0.5 × Tris-borate-EDTA buffer. Electrophoresis was carried out in 0.5 × Tris-borate-EDTA buffer at room temperature at 20 W of constant power for 11 h for segment 3 and 6 h for the other segments (20, 21). Gels were transferred onto Whatman 3MM paper, dried, and autoradiographed with Kodak X-OMAT AR film.

Restriction enzyme studies. 10 U of the appropriate enzyme were mixed with 20 μl of PCR product. The mixture was incubated at 37°C for 4 h (Fok I) or overnight (Nla IV). The digested product was analyzed by agarose gel electrophoresis.

Results

Tritiated fatty acid oxidation by intact fibroblast monolayers. We assayed β-oxidation flux in patients' fibroblasts using [9,10(n)-^{3}H]-palmitate and -myristate as β-oxidation substrates (Table II). [^{3}H]palmitate oxidation measures oxidation flux of medium- and long-chain fatty acids, whereas [^{3}H]-myristate oxidation reflects more sensitively impaired medium-chain fatty acid oxidation. All patients with severe neonatal onset GA II had very low [^{3}H]palmitate oxidations, with values ranging from 2% to 22% of the control mean. [^{3}H]-myristate oxidation was also significantly impaired for these same patients, with values of 2–26% of control. For all but patient 1728, [^{3}H]palmitate oxidation was more impaired than was that of [^{3}H]myristate. All these values are consistent with the severe generalized block in intramitochondrial acyl-coenzyme A dehydrogenation and β-oxidation observed in severe neonatal onset GA II. Late-onset GA II patients 1903 and 1966 yielded distinctly different results. In patient 1903, [^{3}H]-palmitate oxidation approached the normal range (≥ 60% of control) at 55% of control, whereas [^{3}H]myristate oxidation was more clearly abnormal at 31% of control. In patient 1966, both [^{3}H]palmitate and [^{3}H]myristate oxidations were normal at 79% and 107% of control, respectively. These findings are consistent with their mild clinical phenotypes (2).

Table II. ^{3}H-Fatty Acid Oxidation by Fibroblast Monolayers

Substrate:	[9,10(n)-^{3}H]Palmitate	[9,10(n)-^{3}H]Myristate
	pmol/mg prot/h±SEM (% of control)	
Normal controls	2270±432 (100)	3020±511 (100)
Patients		
Neonatal onset		
1430	159±32* (7)	394±87* (13)
1728	499±87* (22)	182±41* (6)
1803	272±69* (12)	788±194* (26)
1863	161±39* (7)	364±75* (12)
1916	45±17* (2)	61±18* (2)
9001	182±53* (8)	667±162* (22)
Late onset		
1903	1250±271* (55)	939±231* (31)
1966	1790±329 (79)	3240±713 (107)

Oxidation of ^{3}H-fatty acids was performed as described earlier, except for the [^{3}H]myristate concentration being 80 μM. Fibroblast cultures from five normal controls and each patient were assayed in duplicate three to five times.

* Difference from control significant at $P < 0.05$.

Immunoprecipitation of ETF subunits. In GA II fibroblasts, we radiolabeled newly synthesized ETF polypeptides and immunoprecipitated the labeled molecules with anti-ETF antiserum. The experiments were repeated independently two to six times for each patient, with consistent results. Two bands of the expected sizes (33 and 30 kD for α- and β-ETF subunits, respectively) were seen after SDS-PAGE of immunoprecipitated samples from control cells (Fig. 1 *A*, lane *5*). These two bands were not seen when an excess of unlabeled pig ETF was added to the immunoprecipitation mixture before the addition of the antiserum or when preimmune serum was used in place of anti-ETF antiserum, confirming that these two bands were indeed the α- and β-ETF subunits. In multiple experiments, both subunits were detected in patients 1728, 1803, 1903, and 1966 with no gross alterations in size or amount of either (Fig. 1 *B*, lanes *1, 3, 7,* and *8*). Although immunoprecipitation of samples from patient 9001 also revealed that both subunits were synthesized, the amount of α subunit synthesized appeared less than in control cells (Fig. 1 *B*, lane *5*). Both subunits were markedly decreased in samples from patient 1863 (Fig. 1 *A*, lane *1*). Neither α nor β subunits were consistently detected in samples from patients 1430 and 1916 (Fig. 1 *A*, lanes *3* and *6*).

PCR amplification and DGGE analysis of preα-ETF gene. GC-clamped DGGE is a rapid and sensitive assay for nucleo-

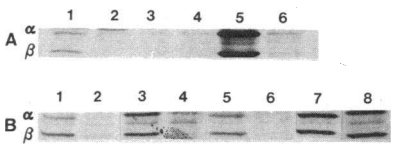

Figure 1. SDS-PAGE of radiolabeled ETF subunits. Confluent monolayers of human fibroblasts were labeled for 12 h with Tran^{35}S-label, solubilized, and immunoprecipitated with anti-ETF antiserum. The immunoprecipitates were electrophoresed on 70% SDS polyacrylamide gels and visualized by fluorography as described previously. (*A*) Lanes *1* and *2*, patient 1863; lanes *3* and *4*, patient 1430; lane *5*, normal control; lane *6*, patient 1916. In lanes *2* and *4*, 6 μg of pure pig liver ETF was added to the cell homogenates before immunoprecipitation. The normal control signal in lane *5* is two- to fourfold denser than usually those seen in normal controls. (*B*) Lanes *1* and *2*, patient 1803; lanes *3* and *4*, patient 1903; lanes *5* and *6*, patient 9001; lane *7*, patient 1728; lane *8*, patient 1966. In lanes *2, 4,* and *6*, 6 μg of pure pig liver ETF was added to the cell homogenates before immunoprecipitation. The range of signal intensities in lanes *1, 3, 5, 7,* and *8* is representative of that found in normal controls.

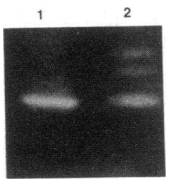

Figure 2. DGGE analysis of segment 2. PCR products were electrophoresed on a 40–65% denaturing gradient gel for 6 h at 60°C and at 150 V. The gel was stained with ethidium bromide and photographed under UV light. Lane *1* shows a sample from a normal control and lane *2* from patient 1803.

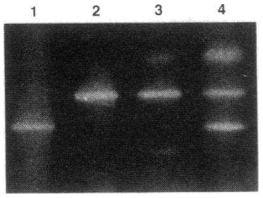

Figure 4. DGGE analysis of segment 4. PCR products were electrophoresed on a 40–65% denaturing gradient gel for 7 h at 150 V and at 60°C. The gel was stained with ethidium bromide and photographed under UV light. Lane *1* shows a sample from a normal control, lane *2* from patient 1728, lane *3* from patient 9001 and lane *4* from patient 1803. A result similar to that displayed in lane *4* was observed in patient 1863.

tide changes, including point mutations (7–9). Samples containing a base change appear on DGGE either as a single band of abnormal migration when homozygous or as multiple bands (usually four bands corresponding to two homoduplexes and two heteroduplexes) when heterozygous (17). The entire coding region of the gene was amplified in five overlapping segments selected to contain only one melting domain when analyzed by perpendicular GC-clamped DGGE. Sequences of the five pairs of primers, size of the amplified product, PCR and DGGE conditions are indicated in Table I. Agarose gel analysis of the amplified products revealed a single band of the expected size in all 13 control and 6 GA II cell lines, except for patient 1916 which showed two bands when segment 5 was amplified, one of the expected size and a second, smaller one. Analysis of the different DNA fragments by DGGE revealed a marked molecular heterogeneity of GA II. The entire procedure (RNA isolation, cDNA synthesis, amplification, and DGGE analysis), was repeated independently at least twice for each segment from each patient, with identical results in each case. Analysis of segment 1 failed to reveal a mutation in any of the eight GA II patients (data not shown). Analysis of segment 2 showed that patient 1803 was heterozygous for a mutation (Fig. 2). This abnormal DGGE pattern was not seen in any of the 13 control or the 7 other GA II patients. Analysis of segment 3 of patient 1430 indicated the presence of two different mutations, one on each allele (Fig. 3). Neither of these changes were present in the other GA II or control samples. However, a different DGGE pattern for segment 3 was found in four of the control cultures, suggesting that they were heterozygous for a normal polymorphism. DGGE analysis of segment 4 indicated the presence of an identical mutation in GA II patients 1728, 9001, 1803, and 1863 (Fig. 4). This mutation was homozygous in patient 1728 and heterozygous in the three other patients. In patient 9001, the data indicated the presence of a different mutation on the other allele within the same segment (segment 4). Analysis of segment 5 showed that patients 1916 and 9001 were heterozygous for different mutations (Fig. 5). DGGE analysis of the five DNA segments from patients 1903 and 1966 did not reveal any nucleotide changes.

SSCP analysis of pre-α-ETF gene. SSCP is a simple and sensitive alternative assay to DGGE for detecting single nucleotide changes (20). The principles of the techniques are different, making them complementary. In SSCP, a labeled single-stranded DNA fragment is electrophoresed on a nondenaturing polyacrylamide gel; a nucleotide change alters its secondary structure and thus its mobility in the gel. SSCP analysis of all five DNA segments did not detect any additional mutations (data not shown). Moreover, SSCP analysis of segment 3 indicated that patient 1430 was heterozygous for only one mutation, missing the mutation in the other allele detected by DGGE analysis within the same segment.

Sequence analysis. The DNA fragments shown by DGGE analysis to contain nucleotide changes were sequenced. Some DNA samples were first purified by agarose gel electrophoresis. In other cases, we separated both alleles on DGGE and sequenced them independently. Sequencing was done at least twice for each patient. In all cases, the DNA sequence obtained exactly matched the published sequence except for the mutations (Fig. 6). The nucleotide changes detected by sequencing in controls and GA II patients are summarized in Table III.

Sequencing of segment 2 from patient 1803 revealed a heterozygous G to A transition at position 346, changing a glycine residue to an arginine at codon 116 (Gly116Arg; Fig. 6 *A*). 4 of 13 controls, but no GA II patients, were shown on DGGE to be heterozygous for a change in segment 3. Sequencing this DNA segment showed a heterozygous C to T transition at nucleotide 512, replacing a threonine with an isoleucine at codon 171 (Thr171Ile; data available on request). DGGE analysis of segment 3 from patient 1430 showed a pattern consistent with the presence of a different base change in each allele. Indeed, sequencing the lowest band revealed an 18-bp deletion (nucleotides 453 to 470), removing six amino acids (codons 152 to 157: del152–157; Fig. 6 *B*). Sequencing the other band showed a T to G transversion at position 470, causing a valine to glycine change at codon 157 (Val157Gly; Fig. 6 *C*). The 18-bp deletion did not allow the formation of heteroduplexes with the allele containing the 470 transversion, consistent with only two homoduplex bands being observed on DGGE.

On the basis of the DGGE analysis of segment 4, the same nucleotide change was present in both alleles from patient 1728 and in one allele from patients 1863, 1803, and 9001. Sequencing the PCR product from patient 1728 revealed homozygous C to T transitions at position 797, replacing a threonine residue

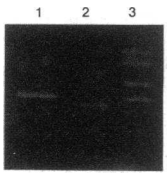

Figure 3. DGGE analysis of segment 3. PCR products were electrophoresed on a 35–65% denaturing gradient gel for 7 hours at 150 V and at 60°C. The gel was stained with ethidium bromide and photographed under UV light. Lane *1* shows a sample from a normal control and lane *2* from patient 1430. Lane *3* shows the DGGE pattern observed in all four control cultures heterozygous for the polymorphism in this segment.

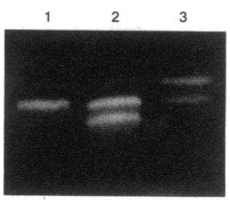

Figure 5. DGGE analysis of segment 5. PCR products were electrophoresed on a 20–45% denaturing gradient gel for 7 h at 150 V and at 60°C. The gel was stained with ethidium bromide and photographed under UV light. Lane *1* shows a sample from a normal control, lane *2* from patient 9001, and lane *3* from patient 1916.

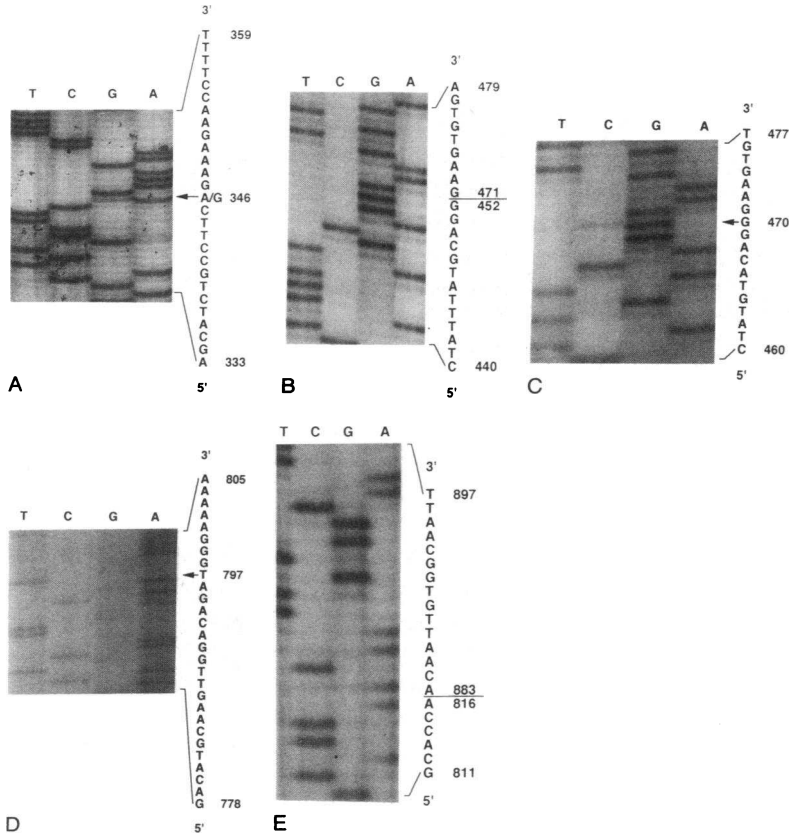

Figure 6. (*A*) DNA sequence of segment 2 amplified from patient 1803's cDNA. The sequence shows a heterozygous G to A transition at position 346. (*B*) DNA sequence of the fragment migrating furthest in DGGE from patient 1430's segment 3. PCR products were electrophoresed on a 45–55% denaturing gradient gel for 7 h at 150 V and at 60°C. The portion of the gel containing the lowest band was excised and the DNA was recovered. The sequence shows an 18-bp deletion from nucleotides 453–470. (*C*) DNA sequence of the fragment migrating slowest in DGGE from patient 1430's segment 3. PCR products were electrophoresed on a 45–55% denaturing gradient gel for 7 h at 150 V and at 60°C. The portion of the gel containing the upper band was excised and the DNA was recovered. The sequence shows a T to G transversion at position 470. (*D*) DNA sequence of segment 5 amplified from patient 1728's cDNA. The sequence shows a C to T transition at position 797. (*E*) DNA sequence of the smaller DNA fragment isolated by agarose gel electrophoresis from segment 5 from patient 1916. The sequence shows a 66 bp deletion encompassing nucleotides 817 to 882.

at codon 266 with a methionine residue (Thr266Met; Fig. 6 *D*). As expected, sequencing the band migrating in the intermediate position on DGGE from patients 1863, 1803, and 9001 confirmed the presence of a homoduplex for this same mutation. Sequencing revealed that the other mutation detected by DGGE in segment 4 from patient 9001 was a deletion encompassing nucleotides 808–810 or nucleotides 809–811, and resulting in the deletion of valine at codon 270 (delVal270; data available on request).

Sequencing of segment 5 from patient 9001 also showed the delVal270 mutation. Sequencing the shorter DNA fragment detected by agarose gel electrophoresis of segment 5 from patient 1916 showed a 66-bp deletion in frame from nucleotides 817–882, resulting in a 22-amino acid deletion from codons 273–294 (del273–294; Fig. 6 *E*). Again, this deletion did not allow heteroduplex formation with the wild-type allele, explaining the presence of only two homoduplex bands on DGGE analysis.

Table III. Pre-α-ETF Polymorphism and Mutations in Controls and GA II Patients

Patients	Alleles	Amino acid change
4 of 13 normal controls	Wild type	None
	C → T 512	Threonine 171 → isoleucine
1430	T → G 470	Valine 157 → glycine
	18 bp deletion (453–470)	Deletion of 6 amino acids (codons 152–157)
1728	C → T 797	Threonine 266 → methionine
	C → T 797	Threonine 266 → methionine
1803	C → T 797	Threonine 266 → methionine
	G → A 346	Glycine 116 → arginine
1863	Wild type	None
	C → T 797	Threonine 266 → methionine
1916	Wild type	None
	66 bp deletion (817–882)	Deletion of 22 amino acids (codons 273–294)
9001	C → T 797	Threonine 266 → methionine
	3 bp deletion 808(9)–810(1)	Deletion of codon 270 (valine)

Restriction endonuclease studies. We designed restriction endonuclease assays to provide an alternative to DGGE analysis for detecting some of the nucleotide changes described. The Thr171Ile change found in four control cell lines removed a Fok I restriction site. As expected, agarose gel electrophoresis of Fok I digested segment 3 DNA from cells without this mutation produced loss of the 382 6bp band (Fig. 7 *A*, lane *3*) and appearance of new bands, consistent with sizes of 178 and 208 bp (Fig. 7 *A*, lane *4*). In contrast, in digested segment 3 DNA from four cultures with this transition, an additional band was seen consistent with a size of 382 bp, corresponding to the uncut homoduplex (Fig. 7 *A*, lane *4*). This result independently confirmed that those four cultures are heterozygous for the Thr171Ile mutation.

Because the del152–157 or the Val157Gly mutations did not create or remove any known cutting sites, we designed an antisense primer to be used with primer 3/1 to introduce a restriction site in this region. The primer was a 25-mer encompassing G471 to G495 in which G474 and T475 were replaced with two C. This modification introduced a Nla IV restriction site when either of the two mutations was present, but not in the amplified product from normal cells. Electrophoresis analysis of the Nla IV digested DNA from patient 1430 confirmed the coexistence of both the del152–157 and Val157Gly mutations (Fig. 7 *B*: lanes *2* and *4*).

Similarly, neither the Thr266Met nor the deletion of codon 270 produced a restriction site change. We designed a 30-mer primer encompassing T747 to A796 in the sense strand, in which A794 was replaced with a G. Amplification with this primer and with primer 4/2 produced a 64-bp fragment containing a Fok I restriction site when the Thr266Met was present in the template. As predicted, products amplified from normal template and analyzed on a 5% NuSieve GTG agarose gel remained intact on Fok I digestion. In contrast, the 64 bp product from patient 1728 was completely cut. Digestion of products amplified from patients 1803 and 1863 only partially cut the 64-bp fragment, confirming heterozygosity for the Thr266Met mutation. Digestion of products amplified from patient 9001 also partially cut the original fragment. However, the uncut fragment was slightly smaller than the uncut fragments from control cells or from patients 1803 or 1863. These data confirm the presence of the Thr266Met change in one allele and of the deletion of codon 270 in the other in patient 9001 (results not shown).

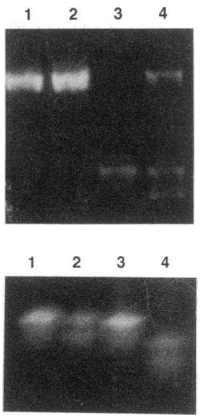

Figure 7. (*A*) Restriction endonuclease analysis of segment 3. Pre-α-ETF cDNA from a homozygous wild-type control cells and from a control heterozygous for the Thr171Ile polymorphism were PCR amplified with primers 3/1 and 3/2 as described in Methods. 20 μl of PCR product was incubated for 4 h at 37°C with 10 U of Fok 1. The undigested (lanes *1* and *2*) and Fok 1 digested (lanes *3* and *4*) products were run on a 4% NuSieve GTG agarose gel. Lanes *1* and *3* show the products derived from the control cells and lanes *2* and *4* from the polymorphic cells. The expected undigested segment 3 size is 382 bp. Digestion of cDNA segment 3 from control cells is predicted to give two fragments of 178 and 208 bp (lanes *3* and *4*). The Thr171Ile polymorphism removes the Fok 1 site, yielding the incomplete cleavage of segment 3 (lane *4*). (*B*) Restriction endonuclease analysis for detection of the del152-157 and Val157Gly mutations. Pre-α-ETF cDNA from control cells and from patient 1430 were PCR amplified with primers 3/1 and a 25-mer antisense primer encompassing G495 to G471 in which C474 and A475 were replaced with two G. 20 μl of PCR products was incubated overnight at 37°C with 10 U of NlaIV. The undigested (lanes *1* and *2*) and digested (lanes *3* and *4*) products were run on a 5% NuSieve GTG agarose gel. Lanes *1* and *3* show the products derived from the control cells and lanes *2* and *4* from patient 1430. The predicted size of both the undigested and digested products from the control cells is 201 bp (lanes *1* and *3*). The heterozygous del152–157 predicts the presence of two bands of 201- and 183-bp sizes after electrophoresis of undigested PCR product amplified from patient 1430 (lane *2*). Because the Val157Gly and del152–157 mutations create a NlaIV site, NlaIV digestion of the PCR product derived from patient 1430 is expected to give two products of 178- and 160-bp sizes (lane *4*) and one of 23 bp (not seen).

Discussion

In this study, we investigated metabolic, polypeptide, and genetic heterogeneity in eight GA II patients. β-Oxidation flux was severely defective in the six early onset GA II patients and intermediate or normal in two patients with late-onset disease. The results of our biosynthetic studies correlated well with both the clinical and metabolic phenotypes. ETF polypeptide levels were decreased or absent in four of the six early onset GA II patients, and normal in the remaining two early-onset patients and the two late-onset patients. We also identified point mutations and deletions in the pre-α-ETF cDNA in all six patients with early-onset GA II, but no mutations in the two late-onset patients.

We can correlate metabolic, polypeptide, and genetic variations in these GA II patients. Patients 1903 and 1966 both presented with a late-onset form of GA II. Patient 1903 had a clinical presentation limited to the neurological system and only excreted small amounts of typical GA II metabolites, whereas patient 1966 had an extremely mild clinical and biochemical GA II presentation. Whereas Loehr et al. (2) could not correlate their residual ETF activities with their clinical phenotypes, our tritiated fatty acid assays clearly separated patients 1903 and 1966 from the other GA II patients. Indeed, although ETF activities in patients 1903 and 1966 were decreased to 6% and 2% of control, respectively, ^{3}H-fatty acid oxidations were intermediate in patient 1903 and normal in patient 1966. We have made analogous observations previously in long-chain acyl-coenzyme A dehydrogenase deficiency, where clinical severities correlated well with [^{3}H]-palmitate oxidations, but not with enzyme activities, suggesting that tritiated fatty acid assays reflect more closely the actual physiologic β-oxidation flux (22). Patients 1903 and 1966 also synthesized normal amounts of α- and β-ETF polypeptides and had no pre-α-ETF cDNA mutations detected by DGGE and SSCP.

The six early-onset GA II patients had generally similar clinical and biochemical phenotypes and very low ^{3}H-fatty acid oxidations in fibroblasts, consistent with their severe GA II phenotypes. In contrast to the biosynthesis studies done by Tanaka and co-workers (3), we radiolabeled the cells for a longer period of time (12 h instead of 1 h). Our results probably reflect the steady-state level of the ETF heterodimer, whereas experiments with shorter radiolabeling periods reflect

synthesis of nascent polypeptides. This experimental difference explains why we did not detect the β subunit in all three patients lacking the α subunit, consistent with the suggestion by Indo et al. (6) that intramitochondrial β-ETF becomes unstable unless bound to α-ETF. Indeed, we detected neither the α nor the β subunits in patients 1430 and 1916, in agreement with the immunoblotting results of Loehr et al. (2), which also reflect ETF steady-state levels. Our finding decreased levels of both α and β subunits in patient 1863 and normal levels of both in patients 1728 and 1803, also agree with the results of Loehr et al. (2). In contrast, the biosynthesis experiments in 1430's fibroblasts by Ikeda et al. (3) demonstrated faint radiolabeling of α-ETF but normal β-ETF biosynthesis. Patients 1430 and 1916 each have one α-ETF allele with in-frame deletions of 6 and 22 amino acids, respectively. This could explain the relative instability of the α-ETF subunit and hence the lack of mature heterodimer in these patients.

We can also make some conclusions regarding the effects of individual point mutations on the stability of the α-ETF subunit. Since patient 1728 is homozygous for the Thr266Met mutation, this mutation, in and of itself, must produce an inactive but stable α-ETF subunit capable of forming a mature heterodimer, since both the earlier immunoblotting studies (2) and our biosynthetic studies show normal amounts of α- and β-ETF subunits in this patient. The Gly116Arg mutation found in one allele of patient 1803 also does not destabilize the α-ETF subunit. Patient 9001 is heterozygous for the Thr266Met mutation and for a deletion of codon 270. It is likely that this deletion produces an unstable α-ETF responsible for the decreased amount of this subunit observed in the immunoprecipitation studies.

GC-clamped DGGE is a sensitive technique for detecting nearly all nucleotide changes. With this technique we identified nucleotide changes in both pre-α-ETF alleles from four patients and in one allele from two early-onset GA II patients. In the two ETF deficient late-onset GA II patients, no base change was detected. None of the identified changes were detected in 13 control cell lines. Moreover, comparison with the published rat pre-α-ETF cDNA sequence (23), modified to include the G likely missing at nucleotide 867 (24), showed that all the amino acids deleted or modified by these changes were conserved amino acids. This suggests that all the nucleotide changes identified are mutations responsible for GA II. The Thr266Met substitution was found in four unrelated patients and appears to be the most common mutation responsible for GA II, whereas the other mutations were identified only in single patients. DGGE and SSCP analyses of segment 4 of patient 1728 indicated homozygosity for the Thr266Met substitution. Since we amplified cDNA and not genomic DNA, we can not exclude the presence of another mutation in the second allele producing an unstable RNA or defective transcription. However, both our immunoprecipitation results and the immunoblotting results by Loehr et al. (2) revealed normal amounts of α-ETF, suggesting that both alleles carry the Thr266Met mutation. We do not know if this patient results from a consanguinous union.

A mutation can be missed by DGGE analysis when two widely separated melting domains are present in the fragment analyzed. If the domain with the higher melting point contains only a few nucleotides, its melting may not produce a marked shift in mobility and its presence may not be detected by DGGE. This situation is illustrated in the DGGE analysis of segment 5 in patients 9001, 1863, 1728 and 1803. Although the C to T transition at position 797 was present in the amplified DNA segment 5 from these four patients, this mutation was not detected by DGGE analysis of this segment. The first bases adjacent to primer 5.1 belong to a small melting domain of much higher melting point than the rest of segment 5. Indeed, perpendicular DGGE of a DNA fragment amplified with primers 4.1 and 5.2 showed the presence of two melting domains with melting points of 53% and 35%. It appears likely that the first bases of segment 5 actually belong to the same melting domain as segment 4. This illustrates the importance of amplifying overlapping DNA segments when performing DGGE analysis.

Patient 1430 has been previously studied by Indo et al. (6). They concluded that this patient (their YH1313) was homozygous for a T to G transversion at nucleotide 470. However, we identified this same mutation in only one allele from this patient, and found an 18-bp deletion in the other allele. Indo et al. devised a PCR method to detect the 470 transversion using an upstream primer encompassing T443 to G469. This primer would have been unable to anneal to the allele containing the 453–470 deletion. This would have resulted in the amplification of only the allele with the transversion, leading them to conclude that patient 1430 was homozygous for the 470 transversion. In patients 1863 and 1916, only one allele appeared to carry a mutation, while the other was identical to the wild type. Other possible explanations is that the undetected mutation involves the regulatory sequences of the gene that we did not analyze or deletions precluding PCR amplification of one or several of our cDNA segments. Alternatively, a mutation in the coding sequence could have been missed by our DGGE assay. DGGE analysis can miss nucleotide changes present in both alleles in which the mutant homoduplex migrates at the same position as the wild type homoduplex. This is illustrated by the G to A transition detected in segment 2 from patient 1803. The wild-type and mutant homoduplexes were only separated with a 10% denaturant gradient, but not with the usual 25% gradient.

In two ETF-deficient late onset GA II patients (1903 and 1966), no changes were detected by our DGGE and SSCP assays. This result can be explained by a mutation located in regulatory sequences or by a mutation in the coding sequence undetected by our assay. Alternatively, these patients may have a mutation in the gene coding for the β subunit of ETF. Although most of the ETF defects involve the α-subunit, some patients have been described with β subunit defects (25). In addition to the mutations detected in GA II patients, we also identified a polymorphism in four normal controls. This polymorphism replaces a threonine for an isoleucine at codon 171. We did not find any cell cultures homozygous for this base change. Because all our control cultures demonstrated normal tritiated palmitate oxidation (data available on request), we can not predict the functional consequences of this nucleotide change being present on both alleles. However, since we found this mutation in 4 of our 13 controls and not in any GA II patients, it is unlikely that this change would produce GA II. Further studies are needed to clarify this point.

We used two other techniques besides DGGE to reveal nucleotide changes in the pre-α-ETF sequence in these patients. SSCP revealed no additional mutations in any segment of this coding sequence. SSCP appeared slightly less sensitive than DGGE, in that it missed the Val257Gly mutation found in

patient 1430, as well as the segment 5 C to T transition at position 797. SSCP analysis also confirmed the DGGE results that only one allele carries a mutation in patients 1863 and 1916. Restriction endonuclease analyses of amplified DNA segments also confirmed the existence of the Thr171Ile mutation in the normal controls, the deletions of codons 152–157 and Val157Gly mutations in patient 1430, both the homozygous and heterozygous Thr266Met mutations in patients 1728 and 1803/1863, respectively, as well as the Thr266Met and the codon 270 deletion in patient 9001. These results confirm the accuracy and sensitivity of DGGE in detecting nucleotide changes responsible for genetic diseases. This simple and sensitive technique allowed us to rapidly screen the pre-α-ETF gene for mutations causing GA II. Identification of mutations responsible for GA II should help our understanding of the interactions between the α- and β-ETF subunits, the various acyl-coenzyme A dehydrogenases and ETF-QO.

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