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Polymorphism of Δ3,5-Δ2,4-Dienoyl-Coenzyme A Isomerase (the *ECH1* Gene Product Protein) in Human Striated Muscle Tissue

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Abstract—Two polymorphic variants of the *ECH1* gene product protein (Δ3,5-Δ2,4-dienoyl-coenzyme A isomerase) have been revealed by proteomics methods in samples of human striated muscle tissue. These variants are identical in molecular weight (29.7 kD) but different in p*I* values (6.57 and 6.75) and in amino acid substitution (41 E→A) confirmed by mass spectrometry. The same type of polymorphism has been detected in samples of different tissues of the same person, so these variants are considered (also based on other data) to be allelic. The rates of these alleles in two representative cohorts of Moscow and Minsk residents are similar.

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The success of the International Human Genome Project, creation of computer interfaces between protein databases, availability of new information on human DNA sequences, as well as qualitative changes in approaches for protein identification due to the development of mass spectrometric methods has opened new possibilities for studies on proteins as gene expression products, this heralding the postgenomic stage of progress in proteomics [1, 2]. All this provides new impetus for studies on polymorphism of different proteins, including those of muscle tissues [2-4].

Proteomic studies on human muscle tissues have already revealed many polymorphic proteins, including those with polymorphism seeming to be due to diallelism of the corresponding genes [2, 3, 5]. This work continues these studies and presents identifications using two-dimensional (2D) electrophoregrams of two allelic variants of the protein product of the *ECH1* gene which, according to the predicted amino acid sequence [6, 7], encodes Δ3,5-Δ2,4-dienoyl-coenzyme A isomerase (DCAI), the enzyme involved in β-oxidation of unsaturated fatty acids [8].

MATERIALS AND METHODS

Autoptates and bioptates of some human striated muscle tissues (myocardium and different skeletal muscles) were analyzed. The autoptates from persons who died because of accidents (with autolysis times of 3-6 h, Moscow residents of the age of 20-60 years, $n = 90$) were obtained from the Forensic Medical Examination Bureau of the Moscow Health Care Department. Bioptates ($n = 25$) resulting from surgical or diagnostic interventions were obtained from the Institute of Experimental Cardiology, Russian Ministry of Health. The samples were usually stored for 1-2 weeks at –70°C before study.

Abbreviations: DCAI) Δ3,5-Δ2,4-dienoyl-coenzyme A isomerase.

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Protein extracts were prepared and fractionated by two-dimensional electrophoresis using our modification of the O'Farrell method; the proteins were visualized by staining with Coomassie Blue R-250 and additionally with $AgNO_3$ if necessary, and the resulting 2D electrophoregrams were analyzed as described in [5, 9]. Densitometry of the 2D electrophoregram fragments under analysis was performed using a demonstration version of the Melanie 3 computer program (GeneBio, Switzerland).

Also analyzed in parallel were 2D electrophoregrams of proteins of myocardium from human embryos and fetuses which had been obtained on investigation of changes in the protein spectrum of myocardium during ontogenesis [10] as part of a work in cooperation with the Institute of Inborn and Inherited Pathology, Ministry of Health of Belarus (the sample of 1990-1991, Minsk).

The treatment of the gels, hydrolysis with trypsin, and extraction of peptides were performed according to protocols [11] with some modifications [12]. The sample (0.5 μl) was mixed on a mass spectrometer subsurface with the same volume of 2,5-dihydroxybenzoic acid (Sigma, USA) solution (10 mg/ml) in 20% acetonitrile supplemented with 0.1% trifluoroacetic acid and dried in air. Mass-spectra were obtained using a Reflex III MALDI-TOF mass spectrometer (Bruker, Germany) with UV laser (336 nm) (positive ion mode in the range of masses from 500 to 8000 daltons). The mass-spectra were internally calibrated using trypsin autolysis products.

Fragmentation mass-spectra were obtained using a MALDI ultraflex mass spectrometer detecting positive ions in tandem mode (TOF-TOF). MALDI fragmentation was enhanced by collision-induced dissociation of the ions caused by admission of helium into the free drift region (the inert gas pressure was $2 \cdot 10^{-6}$ mbar). The error of the fragment mass determination did not exceed 0.05%. The mass spectra presented only signals of the C-terminal peptide fragments broken at the peptide bond (*y*-ions).

The proteins were identified with Mascot software (Matrix Science, USA) using databases of the US National Center of Biotechnological Information. The accuracy of the ion mass determination was 0.01%, and possible modification of cysteine residues by acrylamide and methionine oxidation were taken into consideration.

RESULTS AND DISCUSSION

To continue our long-term systematic studies on human muscle proteins by 2D electrophoresis and other proteomic approaches [2, 5, 9], we have comprehensively analyzed a group of proteins located in one of the central regions of the generalized two-dimensional map. The borders of this region are shown in a typical 2D electrophoregram of heart muscle (Fig. 1). Special attention was given to two protein fractions with the same molecular mass but different p*I* values that displayed characteristic variations in the relative contents in different samples. Thus, on examination of 115 samples of left ventricle muscle, 72 persons were found to have in this region (Fig. 2a) only one protein spot with coordinates of molecular mass/p*I* corresponding to 29.7 kD/6.75 (No. 4472675 according to the nomenclature proposed earlier [5]). In 35 cases, two protein spots (Fig. 2b) were present: No. 4472675 and the spot with coordinates of 29.7 kD/6.57 (No. 4472657). In eight samples (Fig. 2c) only one protein spot with coordinates of 29.7 kD/6.57 (No. 4472657) was found. Based on these observations, it was suggested that such a distribution of the proteins should represent two homozygous and the heterozygous state of the same gene products.

This hypothesis was essentially supported by determinations of relative contents of the proteins Nos. 4472675 and 4472657 in different samples of myocardium. The protein quantities in the corresponding spots were calculated using computerized densitometry and compared with the adjacent reference protein spot (No. 4618682), which displayed no quantitative or qualitative changes in all analyzed cases. In the samples with the supposedly homozygous phenotypes ($n = 10$ and $n = 10$, respectively) the ratio of No. 4618682 to the protein under study was 1 : 0.7. In the samples ($n = 10$) where the two proteins under study were present (Nos. 4472675 and 4472657) the quantity of each of them was 50% decreased (to the ratio of 1 : 0.35), and this represented the effect of the allelic gene dose.

To directly characterize the proteins Nos. 4472675 and 4472657, the appropriate protein spots were cut from

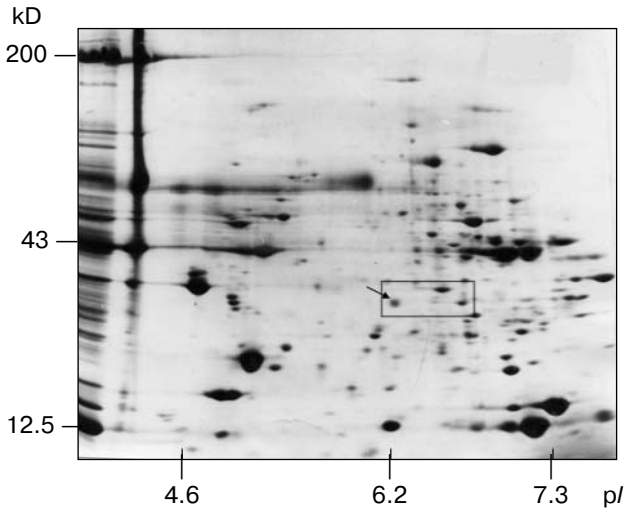

Fig. 1. A typical two-dimensional electrophoregram of proteins from the human heart right ventricle. The zone of a polymorphic protein is delimited by the rectangle. Staining with Coomassie Blue R-250. The abscissa axis: isoelectrofocusing and p*I* values; the ordinate axis: SDS-PAGE and molecular mass (kD).

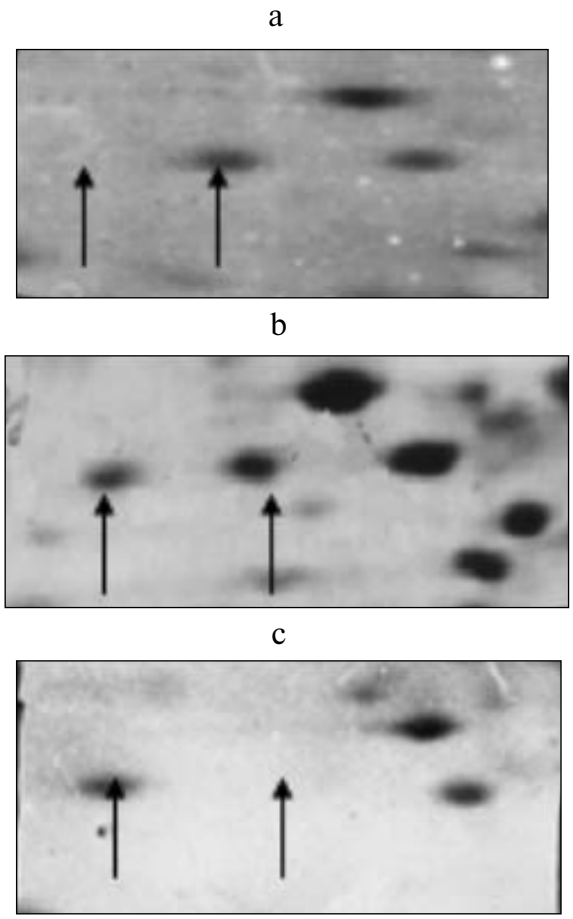

Fig. 2. Fragments of two-dimensional electrophoregrams of human myocardium proteins with polymorphic variants of the *ECH1* gene product protein: a) homozygosity by allele No. 4472675; b) presence of two alleles (heterozygosity): two proteins with coordinates of 29.7 kD/6.75 and 29.7 kD/6.57, respectively; c) homozygosity by allele No. 4472657.

the 2D electrophoregrams obtained for myocardium samples from five persons, two of which were supposed to be homozygous by protein No. 4472675, two others homozygous by protein No. 4472657, and the fifth person seemed to be heterozygous. In fact, three preparations from different persons contained protein No. 4472675 and three preparations contained protein No. 4472657. These proteins were identified using peptides produced by trypsin hydrolysis and found to be products of the same *ECH1* gene believed to encode human Δ3,5-Δ2,4-dienoyl-coenzyme A isomerase (DCAI) and be located on chromosome 19 [6, 7]. The recorded masses of tryptic peptides confirmed 82% of the amino acid sequence of the supposed product of the *ECH1* gene (Fig. 3). Protein No. 4618682 used as the quantitative reference was identified as cytosolic malate dehydrogenase 1.

Based on cDNA sequencing data, polymorphism of the *ECH1* gene products was found earlier [6, 7], with isoforms different in the single amino acid substitution (41 E→A). In the "NCBI protein" database, the corresponding records are presented as Nos. 11433007 and 16924265. These data are in good agreement with our results obtained by mass spectrometry indicating that hydrolyzates of the corresponding proteins contain peptides with the same amino acid substitution manifested by the mass shift of the tryptic peptide with the 33-59 sequence.

Thus, the highly reliable data of mass spectrometry removed virtually all doubts concerning the identification of proteins No. 4472675 and 4472657 (identity of more than 80% of the sequence); however, the experimentally determined values of molecular mass/p*I* of these proteins and the parameters calculated from the nucleotide sequences for the *ECH1* gene products (Δ3,5-Δ2,4-dienoyl-coenzyme A isomerase) were significantly different: 35.8 kD/8.16 for the variant 11433007 and 35.7 kD/8.47 for the variant 16924265. Note that the spot parameters experimentally obtained by two other groups of authors and identified as human DCAI also differed from the calculated values. Thus, the DCAI fraction in the Mito-pick two-dimensional map of mitochondrial proteins (http://www-dsv.cea.fr/thema/MitoPick/Mito2D.html) was characterized by values of 35.9 kD/6.61, whereas data of proteomic analysis of human myocardium proteins resulted in the DCAI parameters of 33.1 kD/6.0 [13].

The discrepancy of calculated and experimental data may be, in particular, explained by existence of a mechanism of alternative splicing and/or posttranslational modifications at the *ECH1* gene expression. A careful analysis of the mass spectrometry data indirectly supports this hypothesis. All mass spectra of tryptic hydrolyzates of these protein spots lacked peaks of the peptides 10-32, 60-65, 98-103, and 159-185 of the calculated amino acid sequence of the *ECH1* gene product (Fig. 3). If peptides 60-65 and 98-103 were undetectable because of limited prescribed parameters of determination, the lack of peptides 10-32 and 159-185 was more likely caused by alternating splicing. On this assumption, the difference between the appropriately calculated value of the molecular mass/p*I* for the *ECH1* gene products and the experimental data virtually disappears.

Rat DCAI was recently shown [8] to have two isoforms, one (36 kD) in peroxisomes and the other (32 kD) in mitochondria. Thus, the human proteins Nos. 4472675 and 4472657 are likely to be just the mitochondrial isoforms of DCAI.

No specific features in the topography of distribution of DCAI isoforms in the myocardium were found (tissues of ventricles, auricles, interventricular and interatrial septa were compared, $n = 5$). Note that polymorphic variants can be detected in myocardium samples from 10 weeks of prenatal development [10], but their amounts are relatively small as compared to the total protein spec-

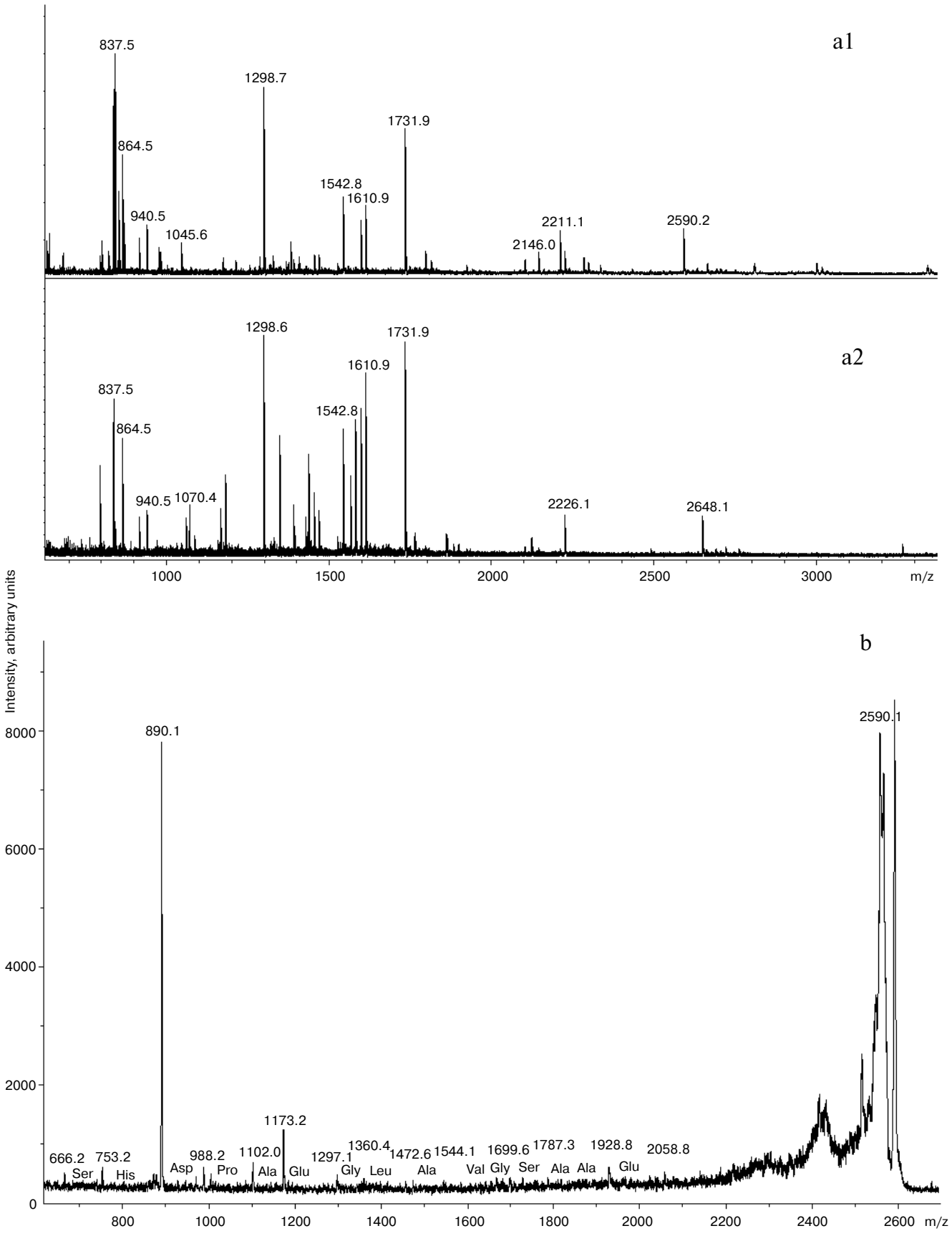

Fig. 3. See figure continuation and legend on p. 452.

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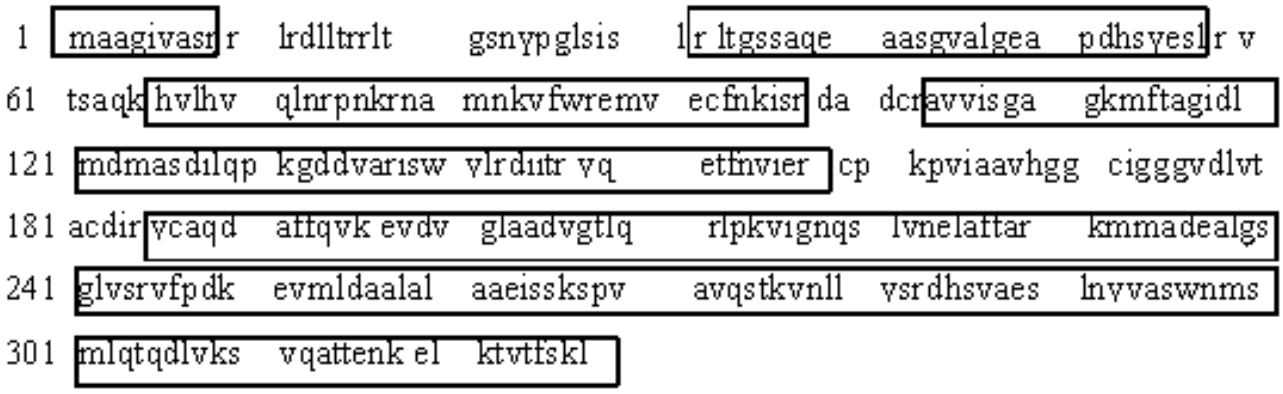

Fig. 3. Mass spectrometric identification of Δ3,5-Δ2,4-dienoyl-coenzyme A isomerase (DCAI) and allelic variants with the amino acid substitution E→A in position 41. a) Peaks of products of trypsinolysis of DCAI allelic variants: a1) contains the tryptic peptide of the 33-59 sequence (m/z 2648.1); a2) the peptide with m/z 2590.2 is detected corresponding to the E→A substitution in position 41. b) Fragmentation mass spectrum of the m/z 2590.2 ion from the tryptic hydrolyzate of the alkaline DCAI variant. The *y*-series of fragmented ions coinciding at 0.05% accuracy with the calculated one for the peptide. c) Amino acid sequence of human DCAI (NCBI protein, record Nos. 11433007 and 16924265); the rectangles delimit the peptides identified by mass spectrometry.

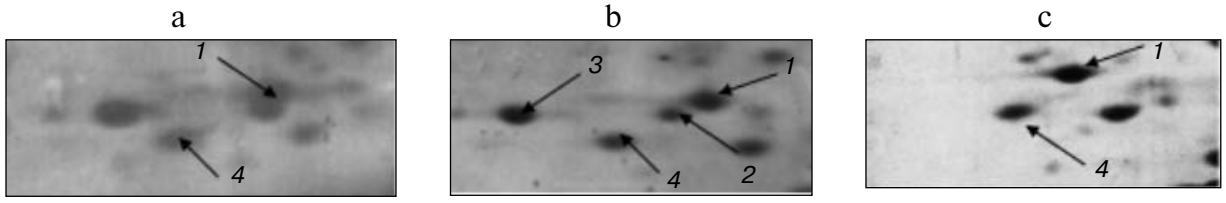

Fig. 4. Fragments (the Δ3,5-Δ2,4-dienoyl-coenzyme A isomerase (DCAI) zone) of two-dimensional electrophoregrams of proteins from different striated muscles: a) human skeletal muscle; b) coelectrophoresis of the skeletal muscle and myocardium; c) human myocardium. The arrows indicate the identified proteins: *1*) malate dehydrogenase; *2*, *3*) isoforms of slow skeletal muscle troponin T1; *4*) DCAI.

trum. According to densitometry of the corresponding 2D electrophoregrams, the ratio of malate dehydrogenase/DCAI was 1 : 0.14 in the myocardium on the 10th week of development, and by the 20-21st week this parameter became stable at the value characteristic for adults.

Samples of human skeletal muscles representing another type of striated muscle tissue were also found to have protein fractions belonging to DCAI with similar properties including the relative contents (Fig. 4). In coelectrophoregrams of myocardium and skeletal muscle tissues (Fig. 4b), DCAI fractions were lower than the fractions identified as slow isoforms of skeletal muscle troponin T1 (record gi:4507625 of database http://www.ncbi.nlm.nih.gov) for which the calculated molecular mass did not exceed 30.0 kD. Note that parallel analyses of the myocardium and skeletal muscle tissue samples from the same person (n = 15) always revealed the same distribution of DCAI isoforms (homozygosity or heterozygosity) in both tissues.

Studies on DNA have already provided data on the rates in different populations of two alleles of the *ECH1* gene, which encode products with the amino acid substitution (41 E→A). For Europeans these rates are characterized by the ratio of 0.292/0.708, for Africans this ratio is 0.783/0.217, and in Chinese it is 0.75/0.25 (from the database on single nucleotide polymorphisms, NCBI SNP rs 9419).

In the present work, the incidence of alleles of this polymorphism was for the first time evaluated by a direct proteomic analysis. The above-presented data on the incidence of two alleles of this polymorphism in the homo- and heterozygous state resulted in the rates of alleles No. 4472657 and No. 4472675 equal to 0.222 and 0.778, respectively for Moscow residents (n = 115), and this virtually corresponds to the parameters for Europeans and close to the rates expected on the base of Hardy–Weinberg equilibrium [14].

Similar studies on a cohort of Minsk residents (n = 98) revealed 14 persons to be homozygous in DCAI iso-

form No. 4472657, heterozygosity was found in 43 persons, and 41 person were homozygous in No. 4472675 isoform. Thus, in the Minsk residents, the rate of the No. 4472657 allele was 0.364 and the rate of the No. 4472675 allele was 0.636. The cohort of Minsk residents consisted of persons of the same generation, whereas the cohort of Moscow residents included representatives of, at least, three generations. Therefore, the small differences in the rates of the DCAI isoforms were likely to be due to both ethnic differences and gene drift [14]. The functional role of the *ECH1* gene polymorphism resulting in products with the amino acid substitution (41 E→A) is still unclear, but the differences in the incidence of the corresponding alleles in different populations suggests that this polymorphism can be associated with specific features of lipid metabolism in humans.

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REFERENCES

1. Jungblut, P. R., Zimny-Arndt, U., Zeindl-Eberhart, E., Stulik, J., Koupilova, K., Pleissner, K. P., Otto, A., Muller, E. C., Sokolowska-Kohler, W., Grabher, G., and Stoffler, G. (1999) *Electrophoresis*, **20**, 2100-2110.
2. Shishkin, S. S., Kovalyov, L. I., and Gromov, P. S. (2000) in *Multiformity of Modern Human Genetics* (Shishkin, S. S., ed.) [in Russian], Gilem, Moscow-Ufa, pp. 17-50.
3. Shishkin, S. S. (2002) *Vestn. Ros. Akad. Med. Nauk*, **4**, 11-16.
4. Shishkin, S. S., Kovalyov, L. I., and Kovalyova, M. A. (2004) *Biochemistry (Moscow)*, **69**, 1283-1298.
5. Kovalyov, L. I., Shishkin, S. S., Efimochkin, A. S., Kovalyova, M. A., Ershova, E. S., Egorov, T. A., and Musalyamov, A. K. (1995) *Electrophoresis*, **16**, 1160-1169.
6. FitzPatrick, D. R., Germain-Lee, E., and Valle, D. (1995) *Genomics*, **27**, 457-466.
7. Strausberg, R. L., Feingold, E. A., Grouse, L. H., Derge, J. G., Klausner, R. D., Collins, F. S., Wagner, L., Shenmen, C. M., Schuler, G. D., Altschul, S. F., et al. (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 16899-16903.
8. Filppula, S. A., Yagi, A. I., Kilpelainen, S. H., Novikov, D., FitzPatrick, D. R., Vihinen, M., Valle, D., and Hiltunen, J. K. (1998) *J. Biol. Chem.*, **273**, 349-355.
9. Pulyaeva, E. V., Kovalyov, L. I., Tsvetkova, M. N., Shishkin, S. S., and Boldyrev, N. I. (1990) *Biokhimiya*, **55**, 489-498.
10. Tsvetkova, M. N., Kovalyov, L. I., Feshchenko, S. P., and Shishkin, S. S. (1992) *Ontogenez*, **23**, 351-363.
11. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Analyt. Chem.*, **68**, 850-858.
12. Govorun, V. M., Moshkovskii, S. A., Tikhonova, O. V., Goufman, E. I., Serebryakova, M. V., Momynaliev, K. T., Lokhov, P. G., Khryapova, E. V., Kudryavtseva, L. V., Smirnova, O. V., Toropygin, I. Yu., Maksimov, B. I., and Archakov, A. I. (2003) *Biochemistry (Moscow)*, **68**, 42-49.
13. Westbrook, J. A., Yan, J. X., Wait, R., and Dunn, M. J. (2001) *Proteomics*, **1**, 370-376.
14. Fogel, F., and Motulsky, A. (1989) in *Human Genetics* [Russian translation], Vol. 1, Moscow, Mir, pp. 152-180.