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M Davidson, A F Miranda, A N Bender, S DiMauro, S Vora

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Research Article

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FIGURE 2

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Muscle Phosphofructokinase Deficiency

BIOCHEMICAL AND IMMUNOLOGICAL STUDIES OF PHOSPHOFRUCTOKINASE ISOZYMES IN MUSCLE CULTURE

MERCY DAVIDSON, ARMAND F. MIRANDA, ADAM N. BENDER, SALVATORE DiMAURO,
and SHOBHANA VORA, *Division of Hematology, Department of Pediatrics and
Departments of Pathology and Neurology, Columbia University College of
Physicians and Surgeons, New York 10032; Department of Neurology,
Mount Sinai School of Medicine, New York 10029*

ABSTRACT Muscle cultures from three unrelated patients with muscle phosphofructokinase (PFK; EC 2.7.1.11) deficiency (Glycogenosis type VII; Tarui disease) had normal PFK activity and normal morphology. Chromatographic and immunological studies showed that normal muscle cultures express all three PFK subunits, M (muscle-type), L (liver-type), and P (platelet-type) and contain multiple homotetrameric and heterotetrameric isozymes. Muscle cultures from patients lack catalytically active M subunit-containing isozymes, but this is compensated for by the presence of P- and L-containing isozymes. Despite the lack of muscle-type PFK activity, presence of immunoreactive M subunit was demonstrable by indirect immunofluorescence, suggesting a mutation of the structural gene coding for the M-subunit of PFK.

INTRODUCTION

Human 6-phosphofructokinase (PFK;¹ ATP: D-fructose-6-phosphate, 1-phosphotransferase, EC 2.7.1.11) is under the control of three structural loci that encode

muscle (M), liver (L), and platelet (P) subunits, which are variably expressed in different tissues. Mature skeletal muscle expresses only the M subunit and contains a single isozyme species, the homotetramer M₄ (1, 2). Random tetramerization of the subunits in other tissues produces various isozymes, which are distinguishable from one another by ion-exchange chromatography or by subunit-specific monoclonal antibodies (1-3). For instance, erythrocyte PFK consists of a five-membered isozyme set composed of M and L subunits, M₄, M₃L, M₂L₂, ML₃, and L₄ (1), whereas leukocyte PFK consists of multiple isozymes composed of all three subunits, with L₄ isozyme predominating (2). Hereditary deficiency of muscle-type PFK (Glycogen Storage Disease type VII; Tarui disease) causes a syndrome characterized by myopathy with exercise intolerance, muscle cramps and myoglobinuria, and hemolysis (4). These patients show total lack of muscle PFK activity, ~50% reduction of the erythrocyte enzyme, and normal activity in leukocytes and platelets. There are conflicting data about the presence of immunoreactive M subunit in muscle (5-9).

Despite the total lack of PFK activity in muscle biopsies, muscle cultures from these patients show normal enzyme activity, normal fusion, and absence of glycogen deposition (10). To elucidate the mechanism underlying the reappearance of PFK activity in muscle cultures, we have studied PFK activity, isozymic profiles, and presence or absence of immunoreactive M subunit in cultured muscle from three unrelated patients with Glycogenosis type VII. Our results show that muscle cultures have normal PFK activity because immature muscle predominantly expresses the P and L subunits, and the presence of P- and L-containing isozymes compensates for the lack of M-containing isozymes.

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¹Abbreviations used in this paper: CRM, cross-reacting material; L, M, and P, liver, muscle, and platelet subunits, respectively; PFK, phosphofructokinase.

METHODS

Patients. Our patients were three unrelated men, 35, 37, and 59 yr of age, with typical clinical and biochemical features of Tarui disease (5, 11). They had a total lack of PFK activity in muscle, half-normal activity in erythrocytes, and normal activity in leukocytes and platelets. Glycogen concentrations in muscle were 1.98, 2.20, and 3.20% (mean \pm SD of 123 controls 1.03 ± 0.18). The residual erythrocyte PFK from each of the three patients consisted exclusively of the L_4 homotetramer; the four M-containing species were absent, indicating homozygosity for the deficiency of the M subunit. The presence of an unstable mutant M subunit was ruled out in each case by the failure to detect M-containing isozymes in reticulocytes separated by density-gradient centrifugation (5, 11).

Muscle cultures. Biopsies were obtained from the right deltoid muscle from each of the three patients after obtaining informed consent. Muscle biopsies were obtained from eight normal individuals during surgical procedures. Muscle explants were planted in 35-mm Costar Cluster dishes (Costar, Cambridge, MA) and grown in a medium with 15% fetal bovine serum as previously described (12). Fibroblasts were selectively removed by "preplating" and transferring myoblast-rich supernatants within 15–20 min to other culture dishes (12). Since fibroblasts rapidly adhere to the substrate, the early transfer effectively eliminates them. Cultures were fed every third day and grown to confluency within ~ 14 d, at which time myoblast fusion was promoted by reducing serum concentration to 8%. Only cultures showing a fusion index (percentage of total nuclei incorporated in myotubes) of 80% or more were used. Creatine kinase isozyme system was used to compare the stage of differentiation of these cultures (12). Muscle cultures from normal individuals and those from the patients were harvested for biochemical studies at about the same stage of differentiation.

Indirect immunofluorescence studies. For indirect immunofluorescence, cultures grown on coverslips were rinsed with phosphate-buffered saline (PBS; pH 7.4) and immediately dried in a stream of air or fixed for 10 s in acetone at 4°C. The cultures were then rehydrated in PBS, drained, and covered with diluted mouse ascites fluid containing monoclonal antibody against the M subunit (1:20–1:200) or against the L subunit (1:200) for 45 min, rinsed with PBS, and then exposed to anti-mouse IgG antibody (1:50–1:100) labeled with fluorescein (Cappel Laboratories, Cochranville, PA). The preparations were washed again thoroughly with PBS, mounted in glycerol/PBS (2:8), and examined and photographed in a fluorescence microscope with epi-illumination, using a BG12 exciter filter and an OG4 barrier filter (E. Leitz Inc., Rockleigh, NJ) with Kodak Tri X film. Parallel cultures were labeled with nonimmune mouse peritoneal ascites fluid (1:50–1:200) instead of monoclonal antibodies or second antibody alone. Muscle cultures treated with non-immune serum and fluoresceinated second antibody (anti-mouse IgG), or treated with fluoresceinated anti-mouse IgG alone showed no fluorescence in nuclei or cytoplasm. The myotubes and mononuclear cells were very faintly outlined in the ultraviolet microscope, making photographic reproduction difficult.

Chromatographic separation of PFK isozymes. Chromatographic separation of isozymes was carried out using DEAE-Sephadex A-25 ion-exchange chromatography at pH 8.0, as described previously (1). Briefly, 1.7×30 -cm column was equilibrated in 0.1 M Tris phosphate (pH 8.0) containing 0.025 M NaCl, 0.2 mM EDTA, 0.2 mM AMP, and 0.7 mM dithiothreitol. The column was loaded with 0.08–0.1 U of

the enzyme preparation. Elution was done with a 300-ml concave gradient of NaCl from 0.025 to 0.525 in the same buffer; 100 fractions were collected (1). The three homotetramers but not the hybrid isozymes are distinguished by their relative positions of elution. M_4 elutes first at 214 mosmol/kg salt, and L_4 elutes last at 480 mosmol/kg salt; P_4 elutes very close to M_4 at 244 mosmol/kg (1, 2). In contrast, the hybrid isozymes of M plus L and P plus L show a wider range of elution, probably due to their different relative amounts in any given PFK preparation. Muscle cultures from six normal individuals and each of the three patients were analyzed chromatographically; some of the cultures were investigated on more than one occasion.

Enzyme-immunoprecipitation studies. The immunoprecipitation studies were done using anti-M (V96-26) and anti-L (V65-06) monoclonal antibodies, and a P subunit-specific mouse heteroantiserum as described previously (2, 3). Since the monoclonal antibodies were found to be nonprecipitating in nature, staphylococci-bearing protein A (IgG-sorb, The Enzyme Center, Boston, MA) were used to precipitate soluble antigen-antibody complexes. Extracts of cultured muscle cells were prepared just before the precipitation studies with chilled 50 mM potassium fluoride (pH 7.5), containing 5 mM EDTA, 5 mM ammonium sulfate, 0.6 mM AMP, 3 mM dithiothreitol, and 0.6 mM fructose 6-phosphate. A given extract was diluted to a concentration of 0.06 U/ml of PFK activity using the extraction buffer and the diluted PFK was distributed in 50- μ l aliquots in a series of eight tubes. 50 μ l anti-M (1:200 in PBS), anti-L (1:200), and anti-P (1:64) antibodies were then added to two tubes each; 50 μ l of non-immune mouse ascites (1:200) or extraction buffer were added to the duplicate control tubes. The mixtures were incubated at 37°C for 30 min and then 100 μ l of freshly washed 10% suspension of IgG-sorb was added.² The mixtures were incubated at 4°C for 30 min with continuous shaking, centrifuged at 9,000 g for 10 min and the supernatants were assayed for residual enzyme activity. The precipitated enzyme activities were expressed as percentages of the concurrent controls; only the precipitation values of $>7.2\%$ were considered to be significant (14). Each extract was tested in duplicate with duplicate controls on two different occasions. Muscle cultures from all eight control subjects and the three patients were investigated by using all three types of antibodies.

Analysis of residual PFK in control muscle cultures after precipitation with anti-M antibody. To investigate whether removal of M_4 and M-containing isozyme(s) in control muscle cultures (using anti-M antibody) would produce an isozyme profile resembling that of the patients' cultures, we obtained ~ 0.06 – 0.08 U of residual PFK, by the following preparative immunoprecipitation experiments. 50 μ l of anti-M antibody (1:50) was added to each of the two 500- μ l aliquots of PFK (0.2 U/ml) from a given normal culture extract. Concurrent analytical immunoprecipitation studies using anti-M antibody (50 μ l of 1:200) and diluted PFK (50 μ l of 0.06 U/ml) were also done as described above to monitor the adequacy of immunoprecipitation in the preparative experiments. Incubation, centrifugation, and precipitation steps were carried out in the standard manner. The residual

² Ig-sorb was washed twice with 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40 (Particle Data Inc., Chicago, IL), 0.02% NaN_3 and then twice with the same buffer but now containing 0.05% Nonidet P-40. It was then suspended to the original volume in the buffer containing 0.05% Nonidet P-40 (13).

enzymes from the preparative precipitation tubes were pooled and subjected to chromatography as described above.

RESULTS

PFK activity and immunoprecipitation values of muscle biopsy specimens and muscle cultures. As shown in Table I, despite a total lack of PFK activity in the muscle biopsies from the patients, their muscle cultures showed normal enzyme activity. Muscle cultures from both normal individuals and patients had approximately threefold higher PFK activity levels than normal muscle.

As previously demonstrated, muscle PFK activity from normal individuals is almost entirely precipitated by anti-M antibody, and is entirely resistant to precipitation by anti-P or anti-L antibodies, indicating exclusive presence of M_4 isozyme. The residual activity in patients' muscle biopsies is too small to allow immunoprecipitation studies. In normal muscle cultures, there is a predominance of P- and L-containing isozymes, and anti-M antibody precipitates only ~15% of total PFK activity. However, the PFK activity of patients' cultures was completely resistant to precipitation by the anti-M antibody, indicating lack of catalytically active M-containing isozymes. The precipitation values obtained using anti-P and anti-L antibodies were similar in cultures from normal and PFK-deficient individuals.

PFK isozymic profiles of skeletal muscle and muscle cultures. Normal muscle PFK eluted as a single peak in the ~40th fraction of the gradient (~214 mosmol/kg) (Fig. 1 A), while normal muscle cultures showed multiple isozymic species (Fig. 1 B); the M_4 peak was distinctly present and represented 15–25% of the total PFK. The enzyme from patients' muscle

cultures consisted of a five-membered set composed of the P and L subunits, i.e., P_4 , P_3L , P_2L_2 , PL_3 , and L_4 ; the M_4 peak was distinctly absent (Fig. 1 C). The P_4 and L_4 peaks are clearly distinguishable from the M_4 peak by their relative positions, in the 47th and 75th fraction during gradient elution (~244 and ~480 mosmol/kg, respectively). The abnormal isozymic profile of the patients' muscle cultures was reproduced upon removal of the M_4 and M-containing isozymes from PFK of control cultures using anti-M antibody (Fig. 1 D).

Indirect immunofluorescence studies of muscle cultures. Despite the lack of catalytically active M-containing isozymes, immunoreactive M subunit was present in mononuclear cells, (including mitotic cells) and multinucleated myotubes from the patients' cultures (Fig. 2). The intensity of the fluorescence was similar to that seen in normal muscle cultures. Use of anti-L antibody also caused intense fluorescence in cultures from both normal individuals and from patients.

DISCUSSION

Since the original description by Tarui et al. (15) and Layzer et al. (16), 21 cases of inherited PFK deficiency in 18 unrelated families have been reported (4). Of these, only nine cases from eight unrelated families exhibited the clinical features of Glycogenosis type VII, simultaneous presence of myopathy and hemolysis; the other cases showed myopathy alone, hemolysis alone, or no symptoms at all. The residual erythrocyte PFK from several individuals with Glycogenosis type VII consisted exclusively of L_4 -type isozyme, indicating a complete deficiency of the M subunit (5, 11). Cultured diploid fibroblasts and Epstein-Barr vi-

TABLE I
PFK Activity and Immunoprecipitation Values of Muscle Biopsy Specimens and Muscle Cultures

| Muscle cells source | PFK activity | Precipitation | | |
|---------------------|--------------|---------------|----------|-----------|
| | | Anti-M | Anti-P | Anti-L |
| | U/g protein | | % | |
| I Biopsy specimens | | | | |
| Normal (12) | 14.85±3.89 | 98.2 | 0 | 0 |
| Patient (3) | 0.2±0.2 | ND | ND | ND |
| II Cultures | | | | |
| Normal (8) | 43±6 | 16±1.8 | 69.8±9.9 | 72.9±10.5 |
| Patient (3) | 39±5 | 0 | 73.8±3.7 | 65.2±2.2 |

ND, not done. Numbers in parentheses represent number of independent cell lines investigated. Data are mean±1 SD.

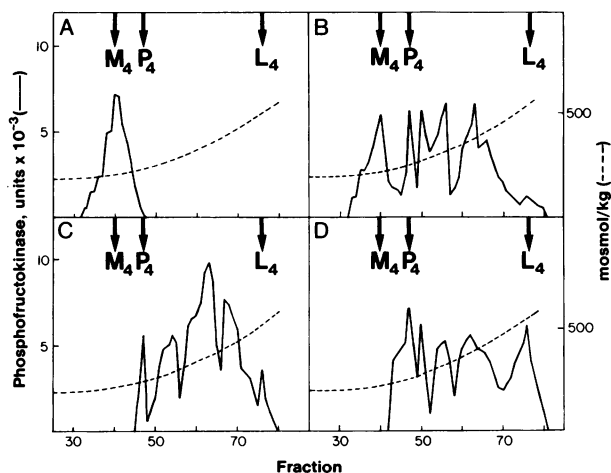


FIGURE 1 Representative chromatographic profile of PFK isozymes from skeletal muscle and muscle cultures. (A), mature skeletal muscle showing the presence of an isolated M_4 isozyme. (B), Normal muscle cultures showing the presence of multiple isozymes (more than five) composed of all three types of subunits (M_4 species is distinctly present). (C), Muscle cultures from patients showing the presence of a five-membered set composed of the P and L subunits only (the M_4 species is distinctly absent). (D), Residual PFK from normal muscle cultures after treatment with anti-M antibody showing a pattern identical to (C).

rus-transformed lymphoblastoid cell lines express all three subunits in similar proportions, and this results in the formation of more than five (usually 7–10), chromatographically distinguishable isozyme species. The deficiency of the M subunit in patients with Tarui disease has been confirmed in these cell types (2, 17): For instance, lymphoblastoid cell lines had half-normal PFK activity, and contained only five isozymes composed of the P and L subunits (2).

Thus, the nature of the enzymatic lesion, total lack of the catalytically active M subunit, is well-established in Tarui disease (5, 11, 17). However, the molecular mechanism of the enzyme deficiency, whether due to total synthetic failure or to synthesis of a mutant M subunit, is uncertain, because only a few cases have been investigated in detail (5, 11, 17). In two unrelated patients, immunologically cross-reactive material (CRM) was absent in muscle (16) and erythrocytes (5), but, CRM was demonstrable in muscle specimens from three other patients (7–9). When mature muscle regenerates in culture, mononuclear muscle satellite cells proliferate and fuse to form multinucleated syncytia (myotubes), thus recapitulating embryonic myogenesis (10, 12). Muscle cultures from two patients showed normal PFK activity, suggesting expression of non-muscle “fetal forms” of PFK *in vitro* (10), but no PFK activity was found in muscle cultures from a third patient with Glycogenosis type VII (9). Therefore, we

have investigated the presence or absence of PFK activity and muscle PFK-specific CRM in muscle cultures from three new patients by using more direct techniques, chromatography, and subunit-specific monoclonal antibodies.

Muscle cultures from these patients showed normal rate of growth and fusion, and normal morphology by light microscopy. Electron microscopic studies in one patient also failed to show glycogen accumulation. In agreement with previous observations (10), PFK activity levels were normal in patients’ cultures (Table I). In fact, PFK activity was significantly higher in muscle cultures from both normal individuals and patients than in normal muscle biopsies. This probably reflects the higher needs for energy and/or glycolytic intermediates of these rapidly dividing cells in culture (4, 18, 19).

Chromatographic and immunologic studies showed that the normalization of PFK activity in patients’ muscle cultures resulted from the expression of the P- and L-type isozymes. In contrast to normal mature muscle, which contains exclusively the M_4 isozyme (Fig. 1 A), normal muscle cultures showed multiple isozymic species composed of all three subunits, indicating expression of the *PFKP* and *PFKL* loci (Fig. 1 B). Immunoprecipitation studies showed that M_4 and M-containing hybrid isozymes represented a relatively minor fraction of total PFK.

Muscle cultures from patients lacked the M_4 isozyme and consisted of the theoretically expected five-membered set that would result from the presence of P and L subunits, i.e., P_4 , P_3L , P_2L_2 , PL_3 , and L_4 (Fig. 1 C). The same five-membered set composed of the P and L subunits was obtained after removal of M_4 and M-containing isozymes from normal muscle culture extracts (Fig. 1 D). The absence of catalytically active M_4 and M-containing heterotetramers was confirmed by the observation that PFK activity in patients’ muscle cultures was totally resistant to precipitation by anti-M antibody. Conversely, immunoprecipitation experiments using anti-P and anti-L antibodies gave similar results in muscle cultures from patients and normal individuals and showed that isozymes containing P and L subunits predominate at all stages of muscle development *in vitro*.

The predominant expression of the P and L subunits by muscle cultures is also observed during ontogeny of muscle *in vivo*; in fetal muscle before mid-gestation, the nonmuscle isozymes predominate, but after mid-gestation, only the M_4 isozyme is expressed (20). It is conceivable that innervation may play a role in this shift of isozyme pattern during myogenesis *in vivo*. This hypothesis can be tested *in vitro*, because human muscle cultures can be innervated by co-cultivation with rodent embryonic spinal cord complex (21). The

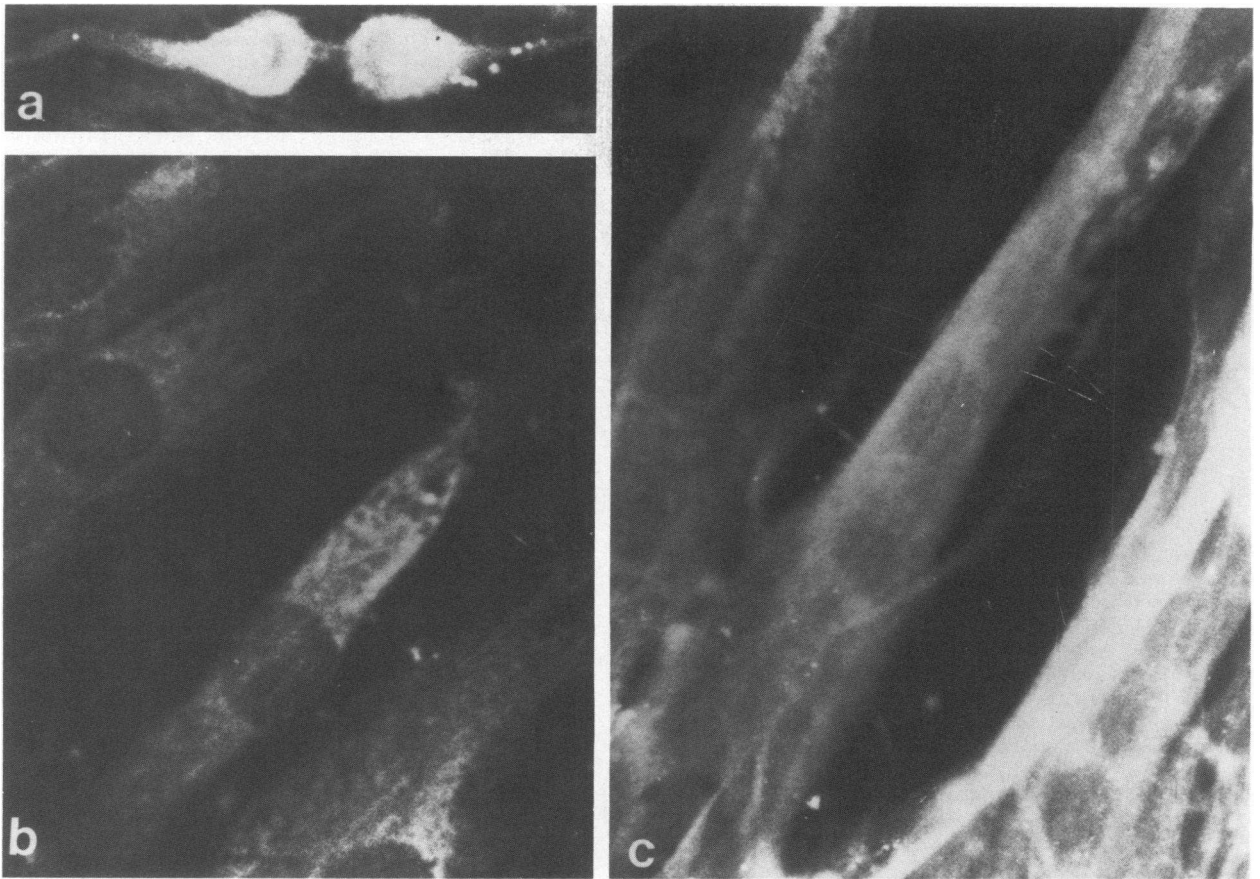


FIGURE 2 Immunocytochemical staining of muscle PFK-deficient muscle cultures using monoclonal anti-M antibody and fluorescein-tagged second antibody (anti-mouse IgG). Presence of immunoreactive M subunits in cytoplasm of mononuclear cells in mitosis (*a*), in interphase (*b*), and in multinucleated myotubes (*c*), of patients' muscle cultures is identical to that seen in muscle cultures from normal individuals. The omission or substitution of the primary antibody with nonimmune mouse serum resulted in a total absence of fluorescence.

expression of P and L subunits in fetal muscle suggests that the similar phenomenon observed in muscle cultures is not due to the presence of exogenous factors in the culture medium, but reflects a difference in genetic program between immature and mature muscle. Developmentally regulated shifts in isozyme patterns during myogenesis have been observed for several other enzymes, such as creatine kinase, phosphorylase, and phosphoglycerate mutase (10, 12), explaining why genetic defects of mature muscle isozymes may not be manifested in muscle cultures (10).

To investigate whether the lack of activity of the M subunit resulted from synthetic failure or from synthesis of a catalytically inactive gene product, we conducted immunocytochemical studies of muscle cultures using monoclonal anti-M antibody. Indirect immunofluorescence showed presence of immunoreactive M subunit in both mononuclear cells and multinucle-

ated myotubes from the patients and the intensity of the fluorescence was similar to that seen in normal muscle cultures (Fig. 2). Thus, the inactive M antigen was demonstrable at all stages of myogenesis *in vitro*. This finding and the demonstration of CRM by immunodiffusion in most patients with Glycogenosis type VII studied so far suggests that the more common genetic lesion is a mutation of the structural gene, rather than a deletion. It is conceivable that the mutation may affect functionally critical parts of the PFK molecule to render it inactive, i.e., the catalytic site, or subunit-subunit interaction sites, since quaternary conformation is essential for enzyme function. Alternatively, other explanations may be entertained, e.g., the absence of a cofactor required for assembly of structurally intact subunits.

In summary, these studies demonstrate that normal muscle cultures express predominantly the P and L

subunits of PFK, and that muscle cultures from these patients with Glycogenosis type VII show no muscle-type PFK activity but have catalytically inactive gene product. The normal differentiation of muscle in vitro despite total deficiency of the muscle-specific PFK is due to the expression of nonmuscle isozymes.

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