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Normal mRNA Content in a Phosphoglycerate Kinase Variant with Severe Enzyme Deficiency

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SUMMARY

A phosphoglycerate kinase variant, PGK-Matsue, was associated with a severe enzyme deficiency, congenital nonspherocytic hemolytic anemia, and mental disorders. The variant enzyme exhibited a slower cathodal electrophoretic mobility and lower affinity toward the substrates. The enzyme activity in the variant's red cells, muscles, and fibroblasts was about 5% of that of normal cells. The content of mRNA in the variant fibroblasts was compared to that of normal cells by the semiquantitative dot hybridization method, and, more accurately, by the liquid hybridization method, using a human PGK cDNA as a probe. It was found that the mRNA level in the variant fibroblasts was comparable to that of normal fibroblasts. The results strongly suggest that the major cause of enzyme deficiency in PGK-Matsue is a seven- to 10-fold increase in the mutant enzyme degradation.

INTRODUCTION

Phosphoglycerate kinase (ATP: 3-phosphoglycerate 1-phosphotransferase, E.C.2.7.2.3, [PGK]) plays a key role for ATP generation in the glycolytic pathway. The enzyme is governed by a gene located on the q-13 position of the human X chromosome [1]. Thus far, 11 rare PGK variants associated with enzyme deficiency have been found in unrelated families (references given in [2]). Many of these deficient variants are associated with congenital hemolytic anemia and mental disorders [2]. The red cell enzyme activity in hemizygous males with these PGK variants ranged from 3% to 20% of normal level [2]. The deficiency was also observed in nucleated tissues, such as the leukocytes and muscles of the subjects [3, 4].

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The complete amino acid sequence of normal human PGK was determined, and specific amino acid substitutions of four PGK variants have been defined (references given in [5]). More recently, cDNA for PGK was cloned [6, 7]. However, the underlying mechanism for PGK deficiency remains obscure. Here, we report quantification of mRNA in normal cells and in variant cells with severe PGK deficiency.

MATERIALS AND METHODS

Fibroblast Cell Lines

The fibroblast cell line from a PGK-Matsue male subject (identification GM0743) and a normal cell line (GM-11) were obtained from the NIGMS Human Genetic Mutant Cell Repository, Camden, N.J. A normal fibroblast cell line from human newborn foreskin (9024) was supplied by Dr. R. Teplitz and Mrs. T. Rundall-Jackson, City of Hope National Medical Center. Another normal fibroblast cell line (K029) was obtained from biopsied skin from an adult male.

The fibroblast cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (Irvine Scientific, Santa Ana, Calif.), 24 mM sodium bicarbonate, penicillin (50 U/ml), and streptomycin (50 mg/ml), at 37°C in 5% CO^2/95% air.

Assay of Enzyme Activity

Harvested cells were extracted with 5 vol of 0.01 M phosphate buffer, pH 7.0, containing 1 mM 2-mercaptoethanol, 1mM EDTA, and 5 μM NADP, by freezing/thawing five times and then centrifuging. PGK and glucose-6-phosphate dehydrogenase (G6PD) activities of the supernatant were assayed by the methods described [8, 9]. Protein was assayed by Lowry's method [10].

Quantification of PGK mRNA by Dot Hybridization

About 10^8 fibroblast cells of passages 13, 14, and 23 were harvested from GM0743, K029, and 9024, respectively. Total nucleic acid (TNA) samples were prepared from each of these cell lines as described [11]. The DNA concentration of the samples was determined as described [12]. TNA samples were treated with supersaturated NaI. The samples were serially diluted in 12.2 M NaI and applied on an mRNC nitrocellulose filter using a manifold apparatus (from Schleicher and Schuell, Keene, N.H.), as described [13]. A full-length cDNA (i.e., 1.8-kb *Bam*HI insert of PGK 825) [14] was radiolabeled to give a specific activity of $2 \sim 3 \times 10^8$ cpm/μg by nick-translation using α[^{32}P]-dATP (500 Ci/nmol, from New England Nuclear, Boston, Mass.) [15] and was used as a PGK cDNA probe. The nitrocellulose filter was prehybridized at 42°C for 12 hrs in a solution containing 40 mM sodium phosphate, pH 6.5, 50% formamide, 5 × SSC (1 × SSC in 0.15 M NaCl, containing 0.015 M sodium citrate, pH 7.0), 5 × Denhardt's solution, 0.25 mg/ml heat-denatured salmon sperm DNA, and 0.1% sodium dodecylsulfate (SDS). The filter was then hybridized at 42°C for 24 hrs in a solution containing 2 mM sodium phosphate, pH 6.5, 50% formamide, 5 × SSC, 1 × Denhardt's solution, 0.1 mg/ml heat-denatured salmon sperm DNA, 0.1% SDS, and PGK cDNA probe. The filter was washed four times for 1 hr each time with a solution of 2 × SSC, containing 0.1% SDS, and then twice for 1 hr each time with a solution of 0.1 × SSC, containing 0.1% SDS, at 50°C. The filter was exposed to Kodak XAR-5 film for 24 hrs.

Quantitation of PGK mRNA by Solution Hybridization

A 30-mer, single-stranded cDNA probe was synthesized according to the PGK cDNA sequence by a solid-phase phosphodiester method (fig. 1). The 30 mer was labeled at the 5′ end with [γ-^{32}P] ATP (6,000 Ci/mmol, from ICN Biochemicals, Irvine, Calif.) and T^4

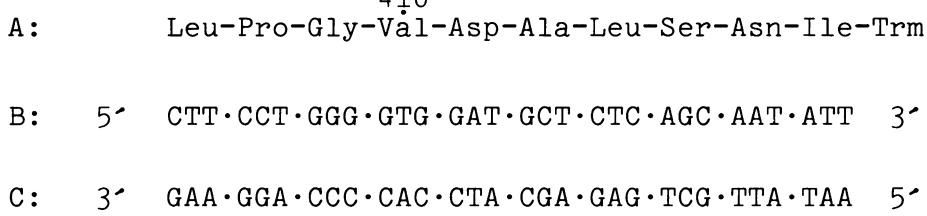

FIG. 1.—Structure of synthetic oliogonucleotide probe. *A*, NH_2-terminal amino acid sequence of PGK; *B*, cDNA sequence of the NH_2-terminal region; *C*, synthetic 30 mer oligonucleotide probe.

polynucleotide kinase (Bethesda Research, Gaithersburg, Md.) by the standard method [16], isolated by urea-polyacrylamide gel electrophoresis, and used as a probe.

The *Bam*HI fragment of PGK 825 was inserted into the replication form M13mp18 [17]. Single-stranded recombinant mp18, which contains the complementary strand of the 30 mer, was selected by the dot hybridization method [18]. The single-stranded DNA was purified from the supernatant of a full-grown infected culture of the selected recombinant twice by the cesium chloride equilibrium centrifugation and used as a PGK mDNA for the quantitation standard [11].

The liquid hybridization was carried out using the radiolabeled 30 mer (12,000 cpm per reaction), by the method of Durnam and Palmiter [11], with the following modifications recommended by Dr. R. D. Palmiter (personal communication, 1984): formaldehyde was replaced by 2 mM Tris-Cl, pH 7.5, containing 0.5 mM EDTA and 0.2% SDS; and sonicated salmon sperm DNA, 100 μg/ml, was included in the dilution buffer. Hybridization was carried out at 82°C for 2 hrs, gradually cooled down to 65°C, and incubated further at 65°C for 10 hrs. The reaction mixture was treated with S1 nuclease, 20 U per reaction (from Bethesda Research) at 37°C for 1 hr. Finally, the hybridized material was precipitated by trichloroacetic acid treatment for 2 hrs at 0°C, and collected on GF/C filters (Whatman). Radioactivity of the precipitate was measured by a liquid scintillation counter. Aliquots (2-, 5-, and 10-μl) of each TNA sample and standard mDNA (ranging from 40 to 2,800 pg) were simultaneously hybridized.

RESULTS AND DISCUSSION

The patient (PGK-Matsue subject, donor of GM0743) suffered from hemolytic anemia and mental disorders and died at age 9 from complications of pneumonia [19].

PGK activity of the patient was severely diminished, that is, less than 5% of normal in both red blood cells and muscle cells [4, 19]. The starch gel electrophoretic mobility of PGK-Matsue was slower than normal [20]. The kinetic properties of PGK-Matsue differed from those of the normal PGK, exhibiting a lower affinity toward the substrates (table 1). The PGK activity of the variant GM0743 was only about 5% of normal cell lines. The ratio between PGK activity and G6PD activity of fibroblast extracts was substantially lower than that of hemoysates, that is, PGK/G6PD was about 9 in normal fibroblasts and about 30 in normal red cells (table 1).

When the content of mRNA in the variant GM0743 was compared to that of the control GM9024 and K029 by the semiquantitative dot hybridization method, no significant difference was found (fig. 2).

More accurate quantification of mRNA content was performed by the liquid

TABLE 1

CHARACTERISTICS OF PGK OF THE VARIANT AND CONTROL CELLS

ORIGIN OF PGK	PGK ACTIVITY		MICHAELIS CONSTANT (μM)			
	U/mg protein	Relative to G6PD	ATP	3PG	ATP	1.3-DPG
GM0743 (PGK-Matsue) ...	0.053	0.5	550	950	250	11.0
GM-11 (control)	1.33	9.5	340	590	...	...
9024 (control)	0.96	8.8	380	550	...	...
K029 (control)	0.79	9.8	...	...	...	...
PGK-Matsue red cells	(0.009)	(1.5)	...	...	...	...
Control red cells	(0.18)	(30.0)	300	620	108	2.4

NOTE: Values in parentheses are activities per mg of hemoglobin. Specific activity of pure normal PGK is 700 U/mg.

hybridization method, using the synthetic 30 mer compatible to PGK cDNA as a probe. To obtain a standard hybridization curve, known amounts of single-stranded mDNA, ranging from 40 to 2,800 pg, were hybridized with the radiolabeled 30 mer. In preliminary experiments, hybridization was carried out at various temperatures (from 37°C to 65°C) for various lengths of time. Under these conditions, however, the results fluctuated, presumably due to formation of a circular form of the single-stranded mDNA. The hybridization condition specified in MATERIALS AND METHODS (i.e., 82°C for 2 hrs, gradual cooling to

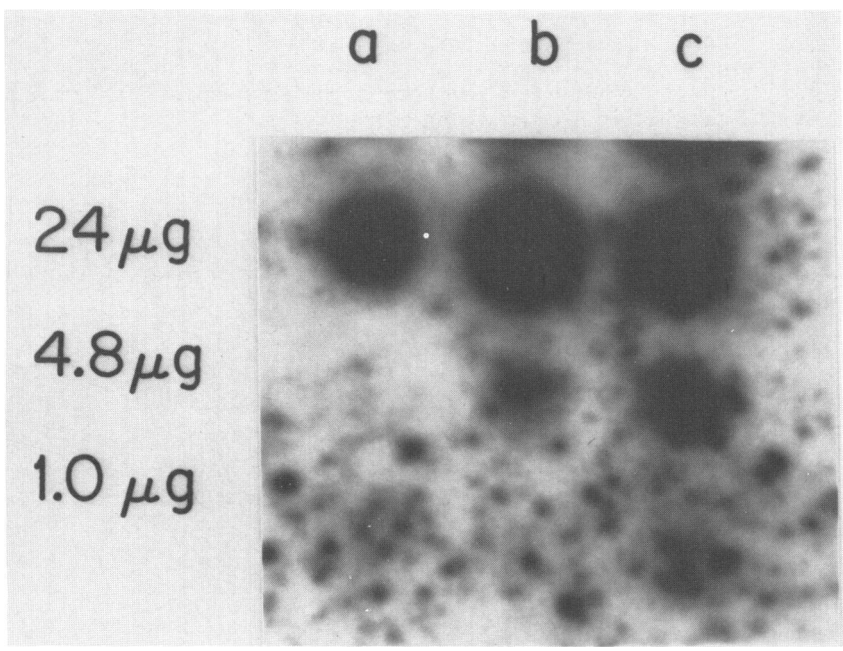

FIG. 2.—Dot hybridization of PGK in RNA. Total nucleic acid samples (TNA), approximately 24 μg, 4.8 μg, and 1.0 μg, were applied to a nitrocellulose filter and hybridized with a PGK cDNA probe. *a*, TNA from 9024 (normal control); *b*, GM0743 (PGK-Matsue); *c*, K029 (normal control).

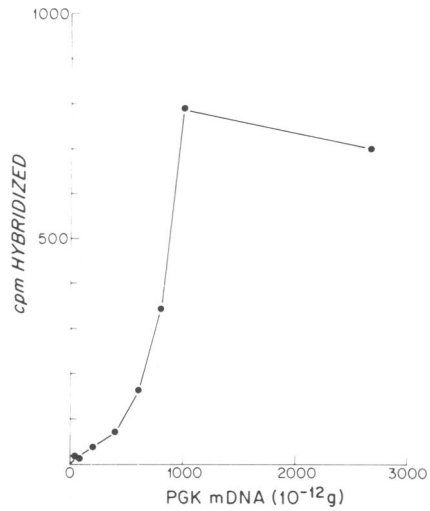

FIG. 3.—Standard curve for solution-hybridization. Data are expressed as radioactivity (cpm) hybridized as a function of PGK mDNA input. The radiolabeled 30 mer (12,000 cpm) was used as a hybridization probe.

65°C, and incubation at 65°C for 10 hrs) gave reproducible results, as shown in the standard hybridization curve (fig. 3). To rule out a possible nonspecific background hybridization, the same amounts of purified human DNA were also treated under the same conditions. In this case, no detectable radioactivity remained after S1 nuclease treatment.

The quantities of PGK mRNA in the variant and control cells are shown in table 2. No marked difference of the PGK mRNA level was found in these three cell lines.

Assuming that mammalian cells contain 10^{-5} μg of RNA per cell, then 1%–5% of total RNA is mRNA [16] and 0.1% of total mRNA is PGK mRNA [14];

TABLE 2

QUANTIFICATION OF PGK mRNA

Cell line	TNA input (μl)	DNA* input (μg)	cpm hybridized	PGK† mRNA (pg)	PGK mRNA (pg)/ DNA input (μg)
GM0743 (PGK-Matsue) ..	2	2.06	14.3	80	38.8
	5	5.15	54.3	300	58.3
	10	10.30	170.3	600	58.3
9024 (control)	2	2.02	10.3	50	24.8
	5	5.05	39.3	220	43.6
	10	10.10	114.3	500	49.5
K029 (control)	2	1.64	7.3	30	18.3
	5	4.10	41.3	230	56.1
	10	8.20	103.3	470	57.3

* DNA content of input TNA.
† Determined using the standard curve (fig. 2).

thus, 2×10^6 cells would contain 200 ~ 1,000 pg of PGK mRNA. Our results are compatible with the calculated figures.

Our study indicates that the mRNA level of the PGK-Matsue fibroblast is not diminished; therefore, the primary cause of the enzyme deficiency in red cells and tissues of the PGK-Matsue subject is not due to a diminished rate of transcription or post-transcriptional processing. Since PGK-Matsue is a structural variant associated with altered electrophoretic mobility and kinetic properties, it presumably resulted from a point mutation in the coding region of the *PGK* locus; therefore, a severely retarded rate of translation of the variant mRNA is unlikely. It is most likely that the major cause of enzyme deficiency in the PGK-Matsue subject is an increased rate of mutant enzyme degradation.

The specific enzyme activity of PGK-Matsue was not directly determined, but it was estimated, using the immunoneutralization test, as being about 35%–40% of normal PGK [20]. Thus, the molecular concentration of PGK-Matsue in the variant cells is about 10%–15% that of normal cells. Assuming that the steady-state enzyme levels can be described by the formula proposed by Schimke and Doyle (i.e., ks = kpE, where ks is the rate constant for synthesis, kp is the rate constant for degradation, and E is the concentration of enzyme at steady state) [21], the rate of degradation of PGK-Matsue should be about 7–10 times higher than that of normal PGK in slow-growing tissues, such as muscle and fibroblast culture.

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