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Molecular Abnormalities of a Human Glucose-6-Phosphate Dehydrogenase Variant Associated with Undetectable Enzyme Activity and Immunologically Cross-reacting Material

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Summary

Among a large number of glucose-6-phosphate dehydrogenase (G6PD) variants associated with different severity of clinical manifestations, enzyme deficiency, and kinetic abnormalities found in humans, only one variant exhibits no measurable activity and lacks an immunologically cross-reacting material in blood cells and other tissues. The mRNA content of the patient's lymphoblastoid cells was found to be normal, and the size of mRNA was also normal (i.e., ~2.4 kb). Western blot hybridization indicated that the patient's cells did not produce cross-reacting material. The variant mRNA was reverse transcribed and amplified by PCR. Nucleotide sequencing of the variant cDNA showed the existence of three nucleotide base changes, i.e., a C→G at nucleotide 317 (counting from adenine of the initiation codon), which should cause Ser→Cys substitution at the 106th position (counting from the initiation Met); a C→T at nucleotide 544, which induces the Arg→Trp at the 182d position; and a C→T at nucleotide 592, which induces Arg→Cys at the 198th position of the protein. The existence of three mutation sites was confirmed by sequencing of selected regions of the variant gene. No base deletion or frameshift mutation was found in the variant cDNA. No nucleotide change was detected in the extended 5′ region, which included the most distal cap site. When the variant cDNA was expressed in *Escherichia coli,* the G6PD activity was ~2% of that expressed by the normal cDNA, and cross-reacting material was undetectable. However, when the variant mRNA was expressed in the in vitro translation system of rabbit reticulocytes, the variant protein was produced. These results suggest that extremely rapid in vivo degradation or precipitation of the variant enzyme induced by the three amino acid substitutions could be the major cause of the molecular deficiency.

Introduction

Glucose-6-phosphate dehydrogenase (G6PD; glucose-6-phosphate; NADP oxide reductase; E.C.1.1.1.49) plays a key role in the generation of NADPH and the production of ribose-5-phosphate, a building block of nucleotide, in the pentose phosphate shunt pathway. Nearly 400 genetic variants—some of which are associated with enzyme deficiency and some of which are associated with abnormal kinetic properties, molecular instability, and altered electrophoretic mobilities—have been reported (reviewed by Beutler and Yoshida 1987; Beutler 1990). Variants with severe activity deficiency are often associated with chronic and drug- and/or food-induced hemolytic anemia. The G6PD abnormalities are the most common cause of these clinical problems.

In all of the severely deficient variants reported, the enzyme deficiency is strongly manifested in unnucleated matured red blood cells (a few percent of normal), but the deficiency is less severe in nucleated tissues (>10% of normal). We previously found a unique G6PD variant in a patient with chronic granuloma and

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hemolytic anemia (Gray et al. 1973). G6PD activity was undetectable not only in the patient's red blood cells but also in leukocytes and fibroblasts, and an immunologically cross-reacting material was undetectable in these tissues. We tried to elucidate the nature of this "null" variant, through (1) examination of the immunologically cross-reacting material by the western blot hybridization, (2) characterization and quantification of mRNA, (3) determination of the nucleotide sequence of the coding region and the extended 5′ region of the variant gene, and (4) expression of the variant cDNA and normal cDNA in the *Escherichia coli* expression system and in the in vitro translation system of rabbit reticulocytes.

Material and Methods

Lymphoblastoid Cell Lines

The patient's lymphoblastoid cells were transformed by Epstein-Barr virus. The transformed normal lymphoblastoid cell line (GM 7254) was obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). The lymphoblastoid cells were cultured in RPMI 1640 supplemented with 10% FCS (v/v) (Gibco), sodium bicarbonate (24 mM), penicillin (50 units/ml), and streptomycin (50 μg/ml) at 37°C in 5% CO_2/95% air.

Enzyme and Protein Assay

Harvested cells were extracted with 2 vol of 10 mM phosphate buffer, pH 7.0, containing 1 mM EDTA, 1 mM 2-mercaptoethanol, and 5 μM NADP, by freezing/thawing five times and then centrifuging. The method used for measurement of G6PD activity has been described elsewhere (Yoshida 1966). The units of activity of the enzyme are defined as micromoles of NADP reduced per minute at 25°C. Protein concentration was assayed by the method of Lowry et al. (1951).

Western Blot Hybridization of Cellular Extracts

The extracts from lymphoblastoid cells were electrophoresed in a polyacrylamide gel containing 0.1% SDS and were transferred to a nitrocellulose filter. The filter was incubated with rabbit anti-human G6PD antibody (IgG) and subsequently with horseradish peroxidase–conjugated goat anti-rabbit IgG (CalBiochem, La Jolla, CA). Antibody binding was visualized by peroxidase staining with hydrogen peroxide and 4-chloro-1-naphthol.

Crude *Escherichia coli* extracts contained proteins which cross-react with the rabbit anti-human G6PD IgG as well as with preimmune rabbit IgG. Therefore, the G6PD expressed in the *E. coli* system was purified through DEAE cellulose (Whatman DE22 and DE52) chromatography and CM-cellulose (Whatman CM52) chromatography, prior to PAGE.

Isolation of Total Cellular RNA and Genomic DNA

Total cellular RNAs were extracted from the variant and control lymphoblastoid cells ($\sim 5 \times 10^7$ cells each) with guanidinium thiocyanate and precipitated by centrifugation in cesium chloride solution. Genomic DNA samples were isolated from leukocytes and cultured lymphoblasts by standard procedures (Sambrook et al. 1989).

Restriction-Fragment-Length Analysis

About 10 μg of genomic DNA was digested by four restriction endonucleases (*Eco*RI, *Hin*dIII, *Kpn*I, and *Pst*I), subjected to a 1.0% agarose gel electrophoresis, transferred onto a nitrocellulose filter, and hybridized with a full-length human G6PD cDNA (RXG5 cloned in this laboratory) in 50% formamide (v/v), 5 × Denhardt's solution, 3 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.4) 0.5% SDS, and 100 μg salmon sperm DNA/ml at 42°C.

cDNA Synthesis and Analysis

The entire coding sequences and untranslated flanking regions of mRNA were reverse transcribed and amplified in eight overlapping segments by using eight sets of sense (A-1, B-1, . . . , J-1) and antisense (A-2, B-2, . . . , J-2) oligonucleotide primers (table 1). In order to create adequate restriction sites, mismatched nucleotides were intentionally included in the primers. The first-strand cDNA copies of mRNA were produced by incubating total cellular RNA (8–10 μg) with one of the antisense primers (A-2, B-2, . . . , J-2; 10 pmol each) and reverse transcriptase, and subsequently the products were amplified by PCR (Saiki et al. 1988). Details of the procedure have been described elsewhere (Maeda and Yoshida 1991). In order to verify the amplified products, one-fifth of each reaction mixture was electrophoresed on a 1.5% agarose gel, transferred onto nitrocellulose filters, and hybridized with the G6PD cDNA probe.

The amplified DNAs were digested with appropriate restriction enzymes whose recognition sites were created by PCR, and they were purified by PAGE

Table 1

Oligonucleotide Sequences

Oligonucleotide[a]	Sequence[b]
A-1	(−6) 5′-GGatCCATGGCAGAGCAGGTGGCCCT-3′ (20)
A-2	(306) 5′-gAAttcGTCCTCCAGCTTGAGCTTCT-3′ (281)
B-1	(248) 5′-GgatCcAGCCCTTCTTCAAGGCCACC-3′ (273)
B-2	(548) 5′-gaattcTCAGAGCTCTGCAGGTCCCT-3′ (523)
C-1	(485) 5′-ggatCcGGAACCGCATCATCGTGGAG-3′ (510)
C-2	(808) 5′-GAattcGCATCTGCAGTAGGTGGTTC-3′ (783)
D-1	(750) 5′-GgATccATTTGGGATCATCCGGGACG-3′ (775)
D-2	(1019) 5′-gAattCGCAAAAGTGGCGGTGGTGGA-3′ (994)
E-1	(974) 5′-ggatCcAGGGTACCTGGACGACCCCA-3′ (999)
E-2	(1262) 5′-gaattCTCCGACTCCTCGGGGTTGAA-3′ (1237)
F-1	(1185) 5′-ggAtccCGAGGCCGTGTACACCAAGA-3′ (1210)
F-2	(1554) 5′-gAattCTCAGAGCTTGTGGGGGTTCA-3′ (1529)
G-1	(−31) 5′-AGGtCGACGACGACGAAGCGCAGA-3′ (−8)
G-2	(134) 5′-TTCTGCAGGTCACCCGATGCACCCAT-3′ (109)
J-1	(1475) 5′-CActCGAGCTGATGAAGAGAGTGG-3′ (1498)
J-2	(1648) 5′-ATcTGCAGCTGAGGTCAATGGTCC-3′ (1625)

[a] Oligonucleotides A-1, B-1, . . . , J-1 correspond to the sense strand of DNA; and oligonucleotides A-2, B-2, . . . , J-2 correspond to the antisense strand of DNA.

[b] Counting from the adenine residue of the initiation codon. Lowercase letters refer to areas altered to create restriction sites (underlined).

followed by electroelution. The purified DNAs were subcloned into pBluescript KS(+) (Stratagene) or M13 vector. DNA sequencing was performed by the dideoxynucleotide chain termination method of Sanger et al. (1977). In order to prevent possible errors in the *Taq* polymerase reaction, DNA fragments isolated from at least two separate amplification reactions were sequenced in both orientations.

Analysis of Genomic DNAs

One-microgram portions of the patient's DNA and of the control genomic DNAs were amplified using the following two sets of primers: (1) 5-a, 5′-GAGgAGCTCAAGCTGGAGGACTT-3′; and 5-b, 5′-CTCcTGCAGGACTCGTGAATGTT-3′; and (2) 6-a, 5′-GCTGGAtCCCGCATCATCGTGGAGA-3′; and 6-b, 5′-GTTCTGCAgCATCTCCTTGCCCAG-3′ (nucleotides indicated by lowercase letters were intentionally mismatched to the G6PD gene to create restriction sites). These primers correspond to a part of exon 5 (5-a, nucleotides 280→303; and 5-b, nucleotides 479→457 [counting from the adenine residue of the initiation codon]) and a part of exon 6 (6-a, nucleotides 488→508; and 6-b, nucleotides 630→607) (Martini et al. 1986). The two PCR products, which contained the mutation sites, were subcloned into pBluescript KS(+) vector and sequenced.

A part of the PCR products produced using the pair of 6-a and 6-b primers was digested with *Hpa*II. Undigested and digested PCR products were subjected to agarose gel electrophoresis, followed by the Southern blot hybridization using G6PD cDNA probe.

Analysis of G6PD mRNA

Total cellular RNA (10 μg), prepared from the normal and variant lymphoblasts, was electrophoresed on a 1% agarose gel containing 2.2 M formamide, transferred onto a nitrocellulose filter, and hybridized with the human G6PD cDNA probe and the human actin cDNA probe which served as an internal reference. One microgram of total cellular RNA was amplified using two sets of primers—i.e., one set of G-1 and G-2 (shown in table 1), specific for G6PD, and another set of 5′-GCGAGCTCGTTGACCGAATCACCGA-3′ and 5′-AACTGCAGAACATCCTTGCCCAGCAG-3′, specific for human phosphoglycerate kinase (PGK) mRNA (Michelson et al. 1983). One-fifth of the PCR products was electrophoresed on a 1.5% agarose gel, transferred onto nitrocellulose filters, and hybridized with the G6PD cDNA probe. The filters were deprobed and rehybridized with the PGK cDNA-825 probe (Singer-Sam et al. 1984).

Examination of 5′ Region of the Gene

The variant and normal genomic DNA samples (200 μg each) digested by *Bam*HI were size fraction-

ated by agarose gel electrophoresis. Fragments (~1.6 kb) which contained exon 1 and exon 2 of the G6PD gene were partially filled-in, ligated with Lambda Zap II vector (Stratagene), and packaged into the competent cells. The resulting libraries from the variant and normal genomic DNAs were screened using the 5′ region (194-bp *Xho*I/*Bam*HI fragment) of G6PD cDNA as a probe. Nucleotide sequences of the inserts of positive clones thus obtained were determined by the standard procedure.

Construction of a Full-Length Variant G6PD cDNA

A full-length variant G6PD cDNA was constructed by replacing a part of the normal full-length cDNA (RXG5), cloned in this laboratory, with the corresponding part of the variant cDNA which included the mutation sites. First, the 5′ region (nucleotides −6 to 479) of the variant mRNA was amplified by PCR using a set of primers, A-1 and 5-b, shown in table 1. Another part (nucleotides 535 to 823) was also amplified using a set of primers, 6-a and 6-c, described above. A part of the open reading frame of the variant cDNA (nucleotides 42 to 815) was constructed by a stepwise ligation of a *Bam*HI/*Acc*I fragment (nucleotides 42 to 436) of the variant cDNA, a *Acc*I/*Sst*I fragment (nucleotides 437 to 534) of the normal cDNA, and a *Sst*I/*Nco*I fragment (nucleotides 535 to 815) of the variant cDNA.

The cDNA clone (RXG5) was digested by *Xho*I, and the insert (nucleotides −153 to 1727) was subcloned into pBluescript II KS(+). The resulting clone was digested by *Bam*HI and *Nco*I, and the *Bam*HI/*Nco*I fragment produced from the insert was eliminated by electrophoresis. A modified pBluescript vector containing 5′ and 3′ regions of the normal G6PD cDNA was thus obtained. Subsequently, the *Bam*HI/*Nco*I fragment (nucleotides 42 to 815) of the variant cDNA was ligated into the modified pBluescript vector. The full-length variant cDNA clone thus obtained was analyzed by mapping with various restriction enzymes and by nucleotide sequencing of the replaced region and its vicinity, confirming the construction of the full-length variant cDNA.

Construction of Expression Plasmid and Expression of Normal and Variant cDNAS in Escherichia coli

To create an *Nco*I restriction site which is adaptable to the expression vector PKK233-2 (Pharmacia), the 5′ region of G6PD cDNA was modified using a set of primers 5′-ACAGCGcCATGGCAGAGCAG-3′ (corresponding to nucleotides −8 to 12) and 5′-GAAGTCCTCaAGCTTGAGCT-3′ (corresponding to nucleotides 303 to 284) (mismatched nucleotides are shown in lowercase letters). The product was digested by *Nco*I and *Hin*dIII, and the resulting fragment (294 bp) was purified and subcloned into *Nco*I/*Hin*dIII-digested PKK233-2. Plasmid DNA prepared from positive clones digested by *Hin*dIII, partially filled in, and was digested by *Bst*EII. The plasmid vector conjugated with a part of G6PD cDNA (nucleotides −1 to 121) was thus produced. A full-length normal G6PD cDNA clone (RXG5) was digested by *Xho*I, partially filled in, and digested by *Bst*EII. The purified fragment containing G6PD cDNA (nucleotides 122 to 1730) thus produced was ligated with the plasmid vector described above and was used for transformation of competent *E. coli* strain DF213 (G6PD-negative mutant, obtained from *E. coli* Stock Center, Yale University).

The plasmid vector ligated with the normal G6PD cDNA was digested by *Bst*EII and *Sst*II, and vector DNA containing the 5′ part (nucleotides −1 to 121) and the 3′ part (nucleotides 988 to 1730) of normal G6PD cDNA was purified. The *Bst*EII/*Sst*II fragment (nucleotides 122 to 987) obtained from the variant cDNA was ligated into this modified plasmid vector, and the product was used for transformation of *E. coli* strain DF213.

The normal and variant cDNAs were expressed in the transformed cells induced with IPTG in their midlogarithmic phase at 37°C for 8 h. Cells (untransformed host cells transformed with the normal or variant G6PD cDNA construct) were each harvested from 1 liter of culture medium, disintegrated by sonication in 2 vol of 0.01 M Tris-HCl, pH 7.0, containing 1 mM 2-mercaptoethanol, and centrifuged. The three extracts, each containing ~350 mg of protein, were used for enzyme assay and, after fractionation, were used for western blot hybridization.

In Vitro Expression of Normal and Variant cDNA

The full-length normal G6PD cDNA clone GF1 obtained in this laboratory (insert nucleotides −9 to 2821, in pBluescript II vector) was digested by *Xho*I, and the 4.7-kb fragment was purified and self-ligated. The resultant clone which contained the entire coding region of G6PD with a short stretch of 3′ nontranslated sequences (nucleotides −9 to 1727) was inserted into *Eco*RI and *Xho*I sites of pBluescript KS II(+). To obtain the expression construct of the variant G6PD cDNA, the *Bst*EII/*Nco*I-digested fragment (nucleotides 121 to 815) of the normal G6PD cDNA construct

was replaced by the corresponding *Bst*EII/*Nco*I fragment of the variant cDNA which contained all three mutation sites. The sequences of both constructs were confirmed by restriction mapping and nucleotide sequencing.

The linearized normal and variant constructs (1 μg each) were transcribed in vitro by T_7 polymerase. The subsequent translation of the capped mRNA was carried out using the rabbit reticulocyte lysate system (Stratagene). The reaction mixture containing 3 μg of the capped mRNA, 2 μl of ^{35}S-methionine (10 μCi/μl; 1,200 Ci/mmol), and 25 μl of the rabbit reticulocyte lysate was incubated at 30°C. Aliquots (5 μl) were withdrawn at 15-min intervals and subjected to SDS-PAGE followed by autoradiography.

Results

G6PD Activity, Cross-reacting Material, and mRNA Content in Cultured Lymphoblasts

The G6PD activity of the normal cellular extract was 0.34 unit/mg protein, while that of the variant cells was undetectable (<0.001 unit/mg). Western blot hybridization of the cellular extracts by using rabbit anti-human G6PD IgG indicated that the variant cells did not contain a detectable amount (<2% of normal) of cross-reacting material (fig. 1). Northern blot hybridization of cellular RNAs, using G6PD cDNA and actin cDNA as probes, indicated that the size of G6PD mRNA (~2.4 kb) and the content of mRNA were similar in the normal and variant cells (fig. 2). Analysis of materials produced by PCR using the specific G6PD primers together with the reference PGK primers confirmed that the G6PD mRNA content of the variant cells was comparable to that of the normal cells (fig. 3).

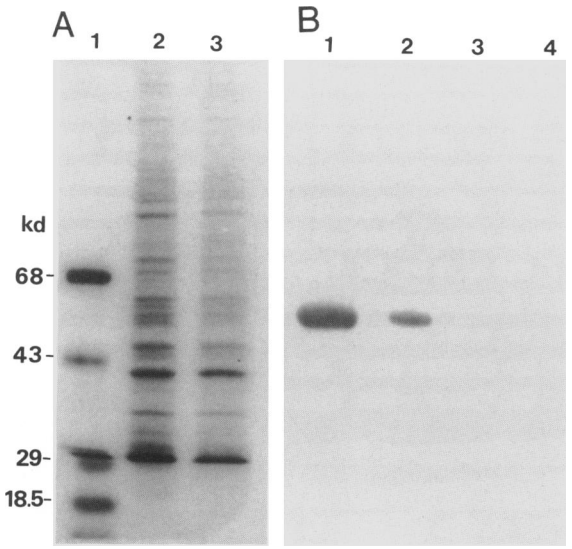

Figure 1 Western blot hybridization of extracts from the normal and variant lymphoblasts. The extracts were subjected to SDS-PAGE (10% acrylamide). *A*, Proteins stained with Coomassie blue. Lane 1, Size-marker proteins. Lane 2, Normal extract (~0.2 mg protein). Lane 3, Variant extract (~0.2 mg protein). *B*, Replica nitrocellulose film stained with rabbit anti-human G6PD IgG and horseradish peroxidase–conjugated goat anti-rabbit IgG. Lane 1, Normal extract (~0.7 mg protein). Lane 2, Normal extract (~0.07 mg protein). Lane 3, Variant extract (~0.7 mg protein). Lane 4, Variant extract (~0.07 mg protein).

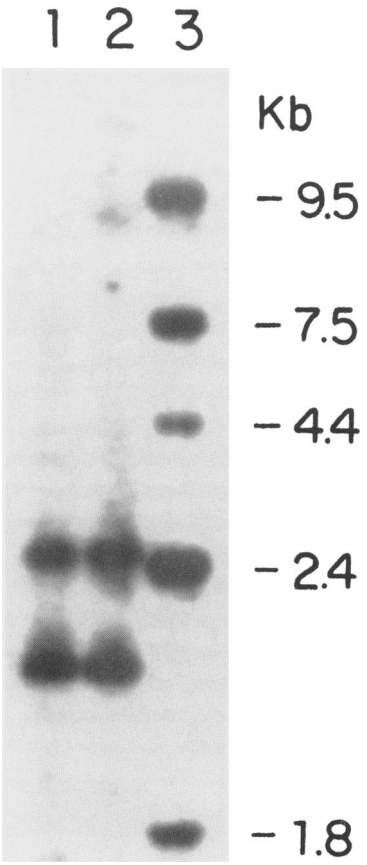

Figure 2 Northern blot hybridization. Total RNAs obtained from the normal and variant fibroblasts were electrophoresed in agarose gel containing formaldehyde and were transferred onto a nitrocellulose filter. The filter was hybridized with a mixture of a full-length G6PD cDNA probe (2.4-kb band) and a reference actin cDNA probe (1.8-kb band). Lane 1, Normal cellular RNA. Lane 2, Variant cellular RNA. Lane 3, Size markers.

Nucleotide Base Changes in the Variant cDNA

Amplification of total cellular RNA by PCR using eight sets of sense and antisense oligonucleotide primers produced eight overlapping cDNA fragments which were hybridizable with the G6PD cDNA and which covered the entire coding sequence. Nucleotide sequence analysis of these amplification products originating from the variant mRNA revealed three nucleotide differences between the variant and the normal G6PD. Mutation regions of the nucleotide sequence ladders of the variant and the normal G6PD are shown in figure 4. The nucleotide base changes are C/G→G/C at nucleotide position 317 (counting from the adenine residue of the initiation codon of mRNA), C/G→T/A at nucleotide position 544, and C/G→T/A at nucleotide position 592. These nucleotide changes should produce amino acid substitutions Ser→Cys at position 106 of the protein (counting from the initiator Met), Arg→Trp at position 182, and Arg→Cys at position 198, respectively.

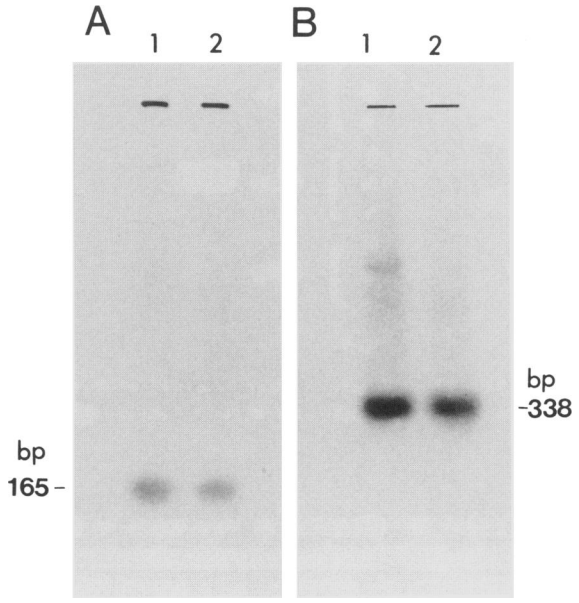

Figure 3 Hybridization of PCR amplification products with specific cDNA probes. The RNA preparations from the normal and variant lymphoblasts were amplified using two sets of primers—one specific for G6PD and one specific for PGK. The products were separated by agarose gel electrophoresis and transferred onto a nitrocellulose filter. The filter was hybridized, deprobed, and rehybridized successively with G6PD cDNA probe (*A*) and PGK probe (*B*). Lane 1, Normal RNA. Lane 2, Variant RNA.

Structure of Variant Gene

No abnormality was observed in the Southern blot hybridization patterns of the variant genomic DNA digested by *Eco*RI, *Hin*dIII, *Kpn*I, and *Pst*I (data not shown). The existence of the three nucleotide base changes in coding exons was confirmed by nucleotide sequencing of the selected genomic regions with the mutation sites (exon 5 and exon 6) produced from the variant genomic DNA by PCR.

The C/G→T/A transition at position 544 is expected to abolish the *Hpa*II cleavage site in the variant gene. In fact, a 143-bp PCR product originating from the variant DNA was not cleaved, while the corresponding product from the normal DNA was cleaved by *Hpa*II (fig. 5). The nucleotide sequences of the extended 5′ region of exon 1, which include the most distal cap site, were found to be identical in the variant gene and the normal gene.

Expression of Variant and Normal cDNAs in Escherichia coli

The variant and normal cDNAs were ligated with the plasmid expression vector PKK233-2. The distance between the ribosome binding site, AGGA, and the initiation ATG is 8 bp, i.e., 5′-. . . CACAGGAAACAGACCATGGGCC . . . -3′.

Extract of *E. coli* cells (G6PD-negative mutant strain) transformed with the plasmid vector ligated with the normal G6PD cDNA exhibited G6PD activity (0.05 unit/mg protein), while the activity in extract of the cells transformed with the variant cDNA was only ~0.001 unit/mg protein. Western blot hybridization of the partially purified cellular extracts by using the rabbit anti-human G6PD IgG indicated that no detectable amount of cross-reacting material existed in the extract of *E. coli* cells transformed with the variant cDNA (fig. 6).

In Vitro Translation of Variant and Normal mRNA

In contrast to the in vivo expression system, the variant G6PD mRNA as well as the normal G6PD mRNA produced a 58-kD protein (fig. 7). Since the rabbit lysate had strong G6PD activity, it is not clear whether the protein produced by the variant G6PD mRNA had catalytic activity.

Discussion

Among nearly 400 human X chromosome–linked G6PD variants reported to date, the variant examined is the only one associated with undetectable enzyme

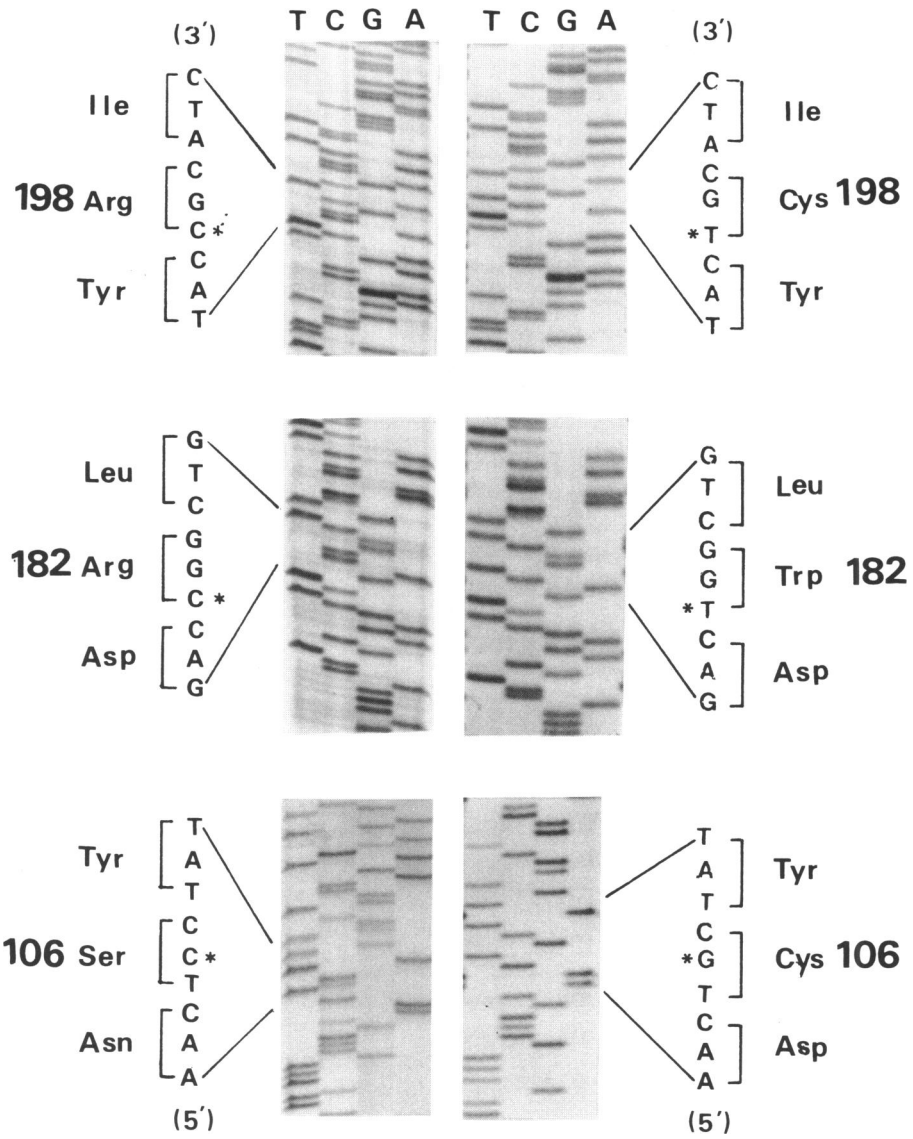

Figure 4 Partial nucleotide sequence of normal G6PD and variant G6PD. The coding sequence is shown aligned with the sequence ladders. Asterisks indicate the substitution sites of the variant gene. The encoded amino acid residues are shown. Numerals correspond to the residue number, counting from the initiation Met of the protein.

activity and immunologically cross-reacting material in red blood cells and other tissues. The detailed clinical features and hematological studies of the propositus and his relatives (Caucasian Canadian) have been reported elsewhere (Gray et al. 1973). In brief, the propositus and his two brothers had chronic nonspherocytic hemolytic anemia, neutrophil dysfunction, and granulomatous lymphadentis. The G6PD activity was undetectable in all three subjects. Their mother's erythrocyte and leukocyte G6PD activity was ~60%–70% of normal. The methemoglobin elution test indicated the presence of two types of red cell populations, and reticulocyte counts were consistently high, suggesting the presence of a mixed hemolytic response in the mother.

Complete (or nearly complete) loss of enzyme proteins would result from (*a*) total or partial deletion of the gene; (*b*) diminished transcription of the structural gene, because of either a defective nonallelic regulatory gene or a defective promoter or intron-exon junction of the gene; or (*c*) formation of a defective primary transcript, associated with nonsense mutation(s),

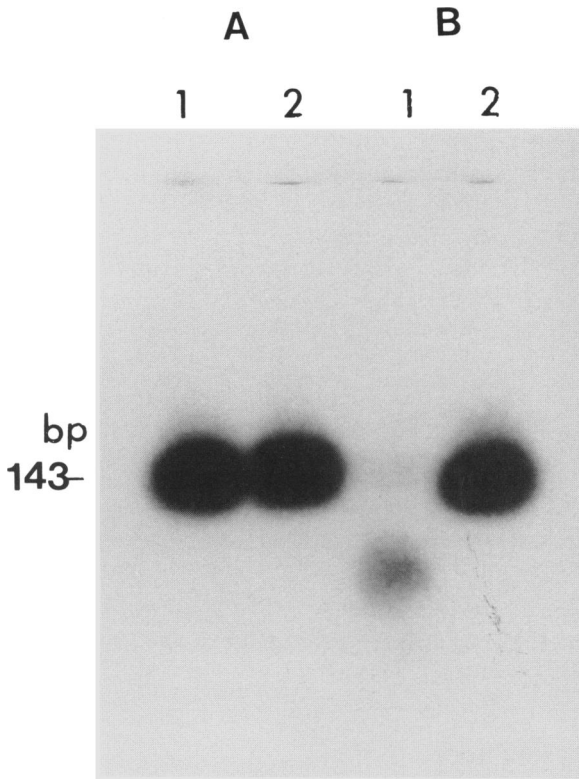

Figure 5 Southern blot hybridization of PCR products of the normal and variant genomic DNAs. The 143-bp genomic region, which includes the C→T transition at nucleotide 544, was amplified using a pair of 5′ and 3′ primers. Half of the amplified products were digested by *Hpa*II. Undigested and digested PCR products were subjected both to agarose gel electrophoresis and to Southern blot hybridization using G6PD cDNA as a probe. *A*, Undigested PCR product; *B*, PCR product digested by *Hpa*II. Lane 1, Normal DNA. Lane 2, Variant DNA.

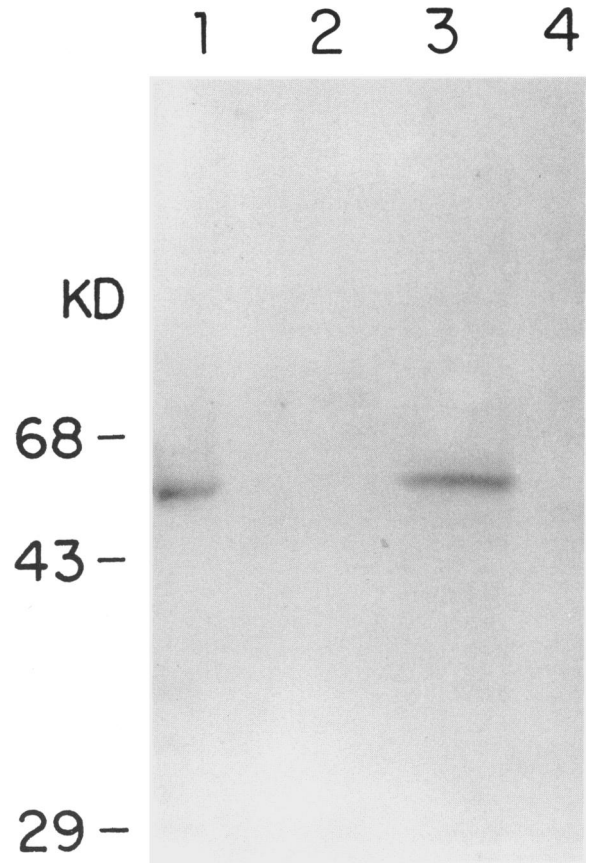

Figure 6 Western blot hybridization of proteins expressed in the *Escherichia coli* expression system. The extract from the host cells transformed with the normal G6PD cDNA construct, as well as that from host cells transformed with the variant G6PD cDNA construct, were fractionated through three steps of chromatography and subjected to SDS-PAGE (10% acrylamide). Lane 1, Partially purified G6PD from red blood cells. Lane 2, Extract of host cells. Lane 3, Extract of cells transformed with the normal G6PD cDNA. Lane 4, Extract of cells transformed with the variant G6PD cDNA.

frameshift mutation(s), or improper splicing site(s) due to mutation(s) of the gene. Examples of such abnormalities have been documented in thalassemias (Orkin 1987), hyperphenylalaninemias (Scriver et al. 1989), and other genetic disorders.

None of the above-mentioned molecular defects can account for the total (or near total) loss of both enzyme activity and enzyme protein in the present variant. The size and the amount of mRNA in the variant cells are comparable to those in normal cells, indicating that the rate of transcription is not diminished in the variant cells. The variant cDNA contains three nucleotide base changes but has no frameshift mutation(s), deletion(s), or nonsense mutation(s).

The most distal cap site is located at nucleotides −166 to −156 from the initiator AUG of G6PD mRNA (A. Yoshida, H. Kanno, and T. Kondo, unpublished observations). The extended 5′ sequence including this cap site is identical in the normal and the variant genes, excluding the possibility of an existence of abnormal ribosome binding site in the variant mRNA. When the normal and variant cDNAs (nucleotides −1 to 1730) ligated with the PKK233-2 vector were expressed in *Escherichia coli* cells, very little G6PD activity (2% of normal) was expressed in the variant system. Since the 5′ ribosome binding site and the 3′ sequence were identical in both expression systems, the observed very low G6PD expression in the variant system is not due to diminished chain initiation or chain termination.

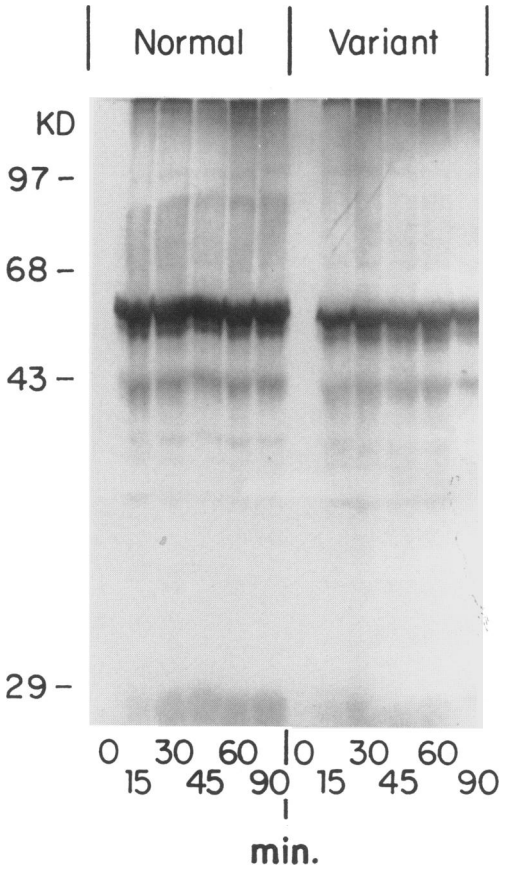

Figure 7 SDS-PAGE (12% acrylamide) of rabbit reticulocyte lysates. The reaction mixtures, taken at different time intervals, were separated in 12% acrylamide gel and subjected to autoradiography.

In marked contrast with the lymphoblastoid cells and the in vivo *E. coli* expression system, the variant G6PD mRNA produced a protein in the in vitro translation system. The size (~58 kD) and amount of the protein produced by the variant mRNA were the same as those produced by the normal G6PD mRNA. The result proves that the variant mRNA does not contain frameshift mutation(s), deletion(s), or nonsense mutation(s).

The variant gene contains three nucleotide base changes which induce three amino acid substitutions—Ser→Cys at the 106th position, Arg→Trp at the 182d position, and Arg→Cys at the 198th position—in the G6PD protein. None of these substitutions is found in other G6PD variants thus far characterized. In view of the commonly accepted concept of mutation, the existence of three missense mutations is quite unusual, and the origin of this variant cannot be readily explained. However, in the present paper, the three mutation sites have been confirmed at the mRNA-cDNA levels and at the genomic DNA level.

Since a protein is produced by the variant cDNA in the in vitro translation system—but barely (or not at all) in the in vivo system—it is most likely that the three amino acid substitutions cause extreme instability in the variant protein and that, in vivo, the variant protein either rapidly degraded or became insoluble. Similar molecular instability was reported in the globin chain variant, $\beta_{Indianapolis}$ (β_{112}Arg) (Adams et al. 1979). Since the present G6PD variant is distinguished from known variants, it is designated as $Gd_{VancouverG317,T544,T592}$.

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