Molecular Heterogeneity of Glucose-6-Phosphate Dehydrogenase A⁻

By E. Beutler, W. Kuhl, J.-L. Vives-Corrons, and J.T. Prchal

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is probably the most common disease-producing genetic polymorphism of humans. Virtually all G6PD-deficient Africans show the G6PD A⁻ phenotype, an electrophoretically rapid, deficient enzyme. The recently acquired ability to identify the point mutations producing the different variants has given us new insights into the population genetics of G6PD variants. Twenty-nine males with the G6PD A⁻ phenotype were studied. They were of African, Mexican, Spanish, and US white ethnic origin. All had the A→G transition at nucleotide 376 characteristic of G6PD A. In each case, one of three additional mutations was present, at nucleotides 202, 680, or 968. That in this population second mutations producing G6PD deficiency occurred only on the genetic background of G6PD A suggests that G6PD A was at one time the most common type of G6PD in Africa. However, the nucleotide sequence of the chimpanzee (*Pan troglodytes*) G6PD indicates that the primordial human type of G6PD was G6PD B.

THE RED BLOOD CELLS (RBCs) and other tissues of most humans contain Glucose-6-phosphate dehydrogenase (G6PD), designated according to its electrophoretic mobility as G6PD B. In Africa, a variant form, G6PD A, occurs with a gene frequency of approximately 0.2.[1] It has normal activity but a more rapid electrophoretic mobility than G6PD B. G6PD A was recently shown to have a mutation at nucleotide 376,[2,3] causing the change from Asn→Asp that characterizes this enzyme and causes its rapid electrophoretic mobility.

G6PD deficiency is common in Africa. The residual enzyme in the African type of G6PD deficiency, like G6PD A, almost always has normal kinetic properties and a rapid electrophoretic mobility. It is designated G6PD A⁻. This type of G6PD deficiency causes susceptibility to the hemolytic effect of drugs[4] and appears to confer resistance to falciparum malaria in female heterozygotes.[5,6]

Recently, we reported the cDNA sequence of four black men and one Mexican man with the G6PD A⁻ phenotype. We found that G6PD A⁻ is characterized by the same mutation that distinguishes G6PD A from G6PD B (ie, A→G at nucleotide 376). In addition, four of the five men had a G→A mutation at nucleotide 202.[7] We have now examined the DNA sequence of G6PD in 29 men with the G6PD A⁻ phenotype and found an unexpected degree of heterogeneity in the basic genetic defect.

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MATERIALS AND METHODS

Blood and DNA samples. Blood samples were obtained from 29 men with G6PD deficiency in whom the residual enzyme was indistinguishable from G6PD A when studied by starch gel electrophoresis in phosphate buffer.[8,9] Enzyme characterization was performed by standard methods.[8] Included were 20 American blacks, two subjects of Mexican and two of Puerto Rican origin, one American white of Northern European origin, and four Spaniards previously diagnosed as having G6PD Betica,[10] a variant that we now realize is identical to the African G6PD A⁻ variant. Blood was obtained from three male *Pan troglodytes* (chimpanzee) from the San Diego Zoo and the Yerkes Regional Primate Research Center (Atlanta, GA). DNA was purified from each sample by standard methods.

Amplification with the polymerase chain reaction and endonuclease cleavage. In each case, the DNA of the portion of exon 5 surrounding nucleotide 376 was amplified with the polymerase chain reaction (PCR).[11] With methods and primers described previously,[7] we determined whether the *Fok*I restriction endonuclease (New England Biolabs, Beverly, MA) could cleave the fragment. Similarly, we examined the sequence in the area of nucleotide 202 in exon 4 by PCR amplification and digestion with the *N1a*III restriction endonuclease (New England Biolabs) to demonstrate the G→A transition. The mutations found at nucleotides 680 and 968 could also be identified by restriction endonuclease digestion of genomic DNA fragments amplified by PCR. The primers used and fragments obtained are summarized in Table 1.

Sequence analysis. In cases in which the G→A transition at nucleotide 202 was not present, all exons were amplified by PCR. The primers used to amplify exons 3 through 10 were designed from the intron sequences we had determined on genomic clones.[12] The flanking sequences surrounding exons 1 and 2 were obtained from Dr Akira Yoshida. The amplified fragments were sequenced using the chemical cleavage method,[13] and were compared with the known cDNA sequence.[14,15]

The G6PD of RBCs from three male *Pan troglodytes* (chimpanzees) were studied by starch gel electrophoresis. DNA was purified from the blood of one chimpanzee whose G6PD had fast electrophoretic mobility and from the blood of one with slow electrophoretic mobility. Exon 5 (containing nucleotide 376) was amplified using the same flanking primers used to amplify the human enzyme. Sequencing was performed by the chain termination method,[16] using Taq polymerase (Promega, Madison, WI) after the appropriate band was reamplified.

RESULTS

All 29 subjects studied had the A→G mutation at nucleotide 376, as revealed by digestion of the amplified genomic DNA fragment with *Fok*I. Twenty-six of the subjects also had a G→A mutation at nucleotide 202, the mutation we

Table 1. Detection of Mutations by Endonuclease Cleavage After PCR

Mutation	Sense Primer	Antisense Primer	Restriction Endonuclease	Uncut	Fragment Size (bp)	
					Cut (Normal)	Cut (Mutant)
202 G→A	5'-GTGGCTGTTCCGGGATGGCCTTCTG	3'-GTTTGTCTCACTCGGGAAGAAGTTC	*Nla*III	109	109	63, 46
376 A→G	5'-TGGCCAGTACGATGATGCAG	3'-TGGCGGAGAAGATGGACCGG	*Fok*I	90	90	58, 32
680 G→T	5'-ACATGTGGCCCCTGCACCAC	3'-GTCCCACCGTCTCGTCAGTG	*Bst*NI	242	213, 29	98, 115, 29
968 T→C	5'-TCCCTGCACCCCAACTCAAC	3'-CGGGTCGTTCCGTCTTGACC	*Nci*I	282	282	162, 120

had previously found in four of five men with the G6PD A⁻ phenotype (Fig 1). As shown in Fig 2, two other mutations were found, a T→C change at nucleotide 968 producing a Leu→Pro substitution, and a G→T at nucleotide 680 causing an Arg→Leu substitution. Because these changes produced new restriction sites, their presence could be confirmed by amplifying the relevant portion of the DNA and cleaving with the appropriate restriction endonuclease (Table 1 and Figs 3 and 4). Distribution of the three mutations is summarized in Table 2.

In some cases, enough blood could be obtained to permit biochemical characterization of the sample. The results of these studies are summarized in Table 3. Although the Spanish sample (G6PD Betica) with the mutation at nucleotide 968 had characteristics indistinguishable from those observed when the mutation was at nucleotide 202, the G6PD obtained from the black subject with this mutation that had been designated G6PD Selma had quite different enzymatic characteristics. The latter sample was characterized twice with essentially equivalent results. Unfortunately, insufficient amounts of blood were available from the subject with the mutation at nucleotide 680 to allow its characterization, and we have been unable, despite repeated efforts, to obtain additional blood from this individual.

Exon 5 and flanking introns of chimpanzee genomic DNA was amplified with PCR. The amplified fragments were treated with the restriction endonucleases *Fok*I and *Bsm*I. The former endonuclease cleaves the G6PD A sequence, and the latter cleaves the G6PD B sequence. Cleavage occurred with *Bsm*I but not with *Fok*I. In addition, the amplified segment was sequenced (Fig 5). Apparently, chimpanzee electrophoretically normal G6PD (B-like) and rapid (A-like) sequences are the same as G6PD B at nucleotide 376 (Fig 5), confirming the results obtained by restriction enzyme analysis. The three nucleotide substitutions in chimpanzee G6PD are all in silent positions. The portion of the intron sequenced is also identical except for the insertion into the chimpanzee of a heptameric repeat of a normal sequence.

DISCUSSION

G6PD A⁻ has previously been considered to be quite homogeneous, and the molecular variability we found is surprising. It is not that different mutations are found in different population groups. Indeed, the limited sample that we examined shows similarity between the Spanish and the African groups, and the mutation at nucleotide 202 was also reported to occur in an Italian male.[3] Rather, within each group several different variants were found, all three in blacks.

Whether these variants differ kinetically is not clear. We

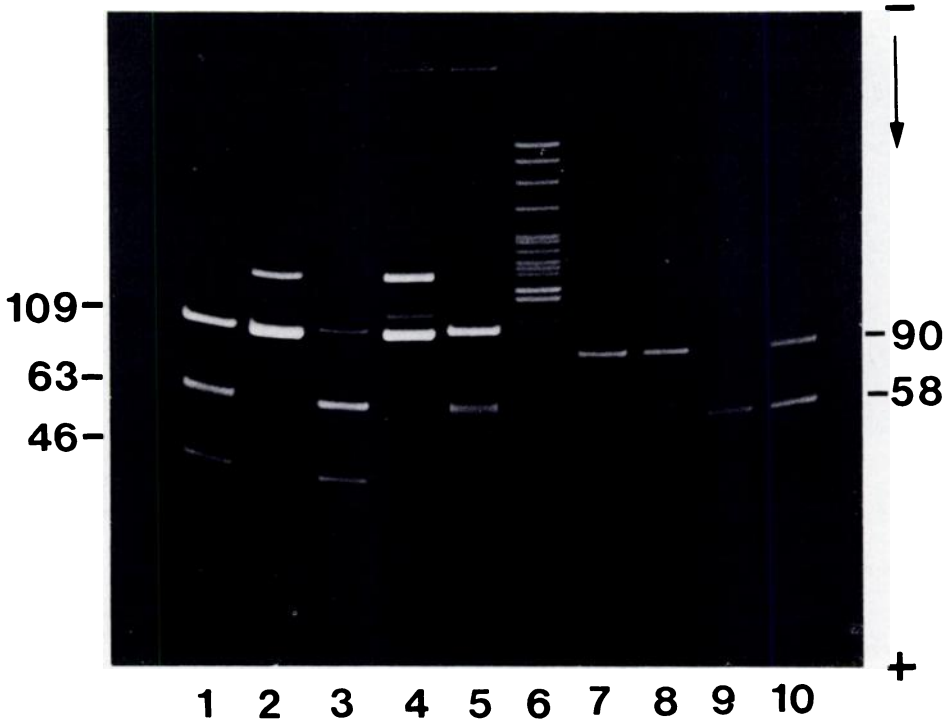

Fig 1. Restriction endonuclease analysis of DNA for the 202 G→A and the 376 G→A mutations. Channels 1 through 5 were tested for the mutation at nucleotide 202; channels 7 through 10 were tested for the mutation at nucleotide 376 as indicated in Table 1. Channel 6 contained pBR322 digested with *Msp*I, serving as a size marker. The DNA samples in channels 1, 3, 5, 9, and 10 were from subjects with G6PD A⁻ $^{202A/376G}$ cut with the endonuclease indicated above. Channels 2, 4, and 8 contained amplified DNA samples not treated with endonuclease. The sample in channel 7 was G6PD B. The 180-base pair (bp) fragment in channels 2 and 4, which disappeared on endonuclease cleavage, was an artefact found in all samples and presumably represented an unrelated area in the genome to which the primers anneal.

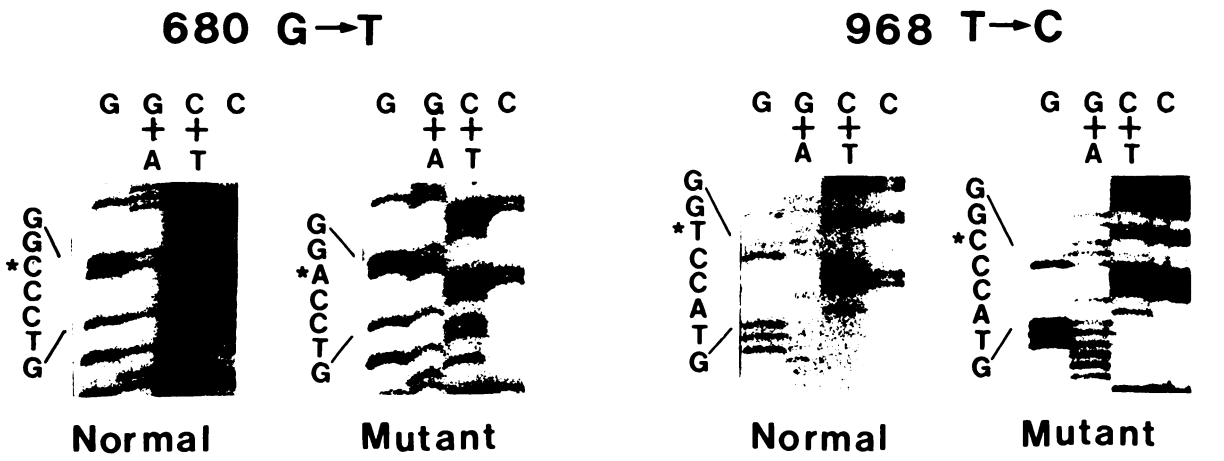

Fig 2. Autoradiograph of sequencing gels of normal and mutant DNA sequenced after PCR of genomic DNA followed by chemical cleavage (described in text). The nucleotide mutation at 680 is shown in the antisense strand reading from nucleotide 678 to 685, therefore showing the mutation as C→A. The T→C mutation at nucleotide 968 is shown in the sense strand reading from nucleotide 970 to 963. *indicates the site of the mutation.

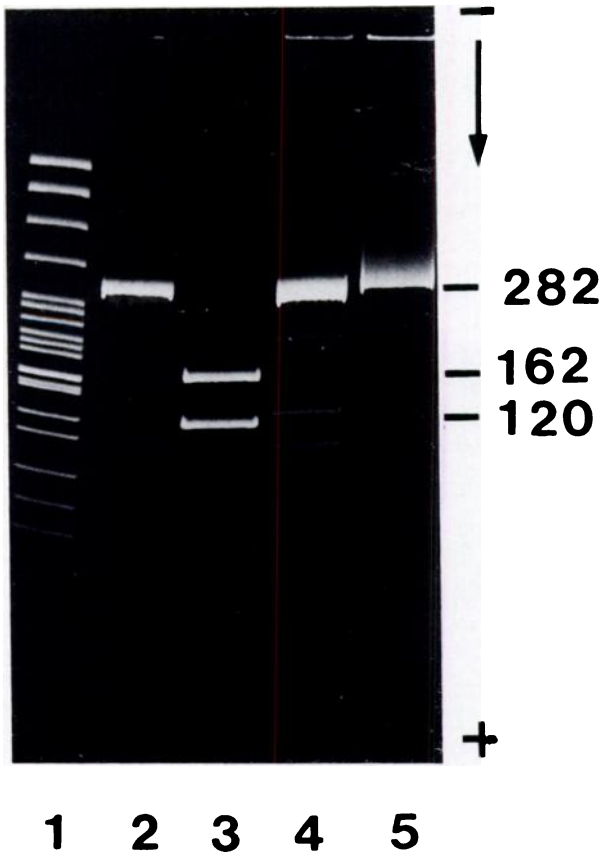

Fig 3. *Nci*I cleavage of the 282 bp PCR fragment of G6PD containing exon 9. A new *Nci*I site is created by the T→C mutation at nucleotide 968, resulting in two fragments of 120 and 162 bp. Channel 1, pBr322/*Msp*I size markers; channel 2, G6PD $A^{-376G/968C}$, uncut; channel 3, G6PD $A^{-376G/968C}$, cut; channel 4, G6PD B, uncut; channel 5, G6PD B, cut.

originally reported unique kinetic properties in the variant lacking the mutation at nucleotide 202.[7] This black man (designated as having G6PD Selma) is included in the present study and had the mutation at nucleotide 968. However, a subject who had been characterized previously as having G6PD Betica had the same mutation. The latter

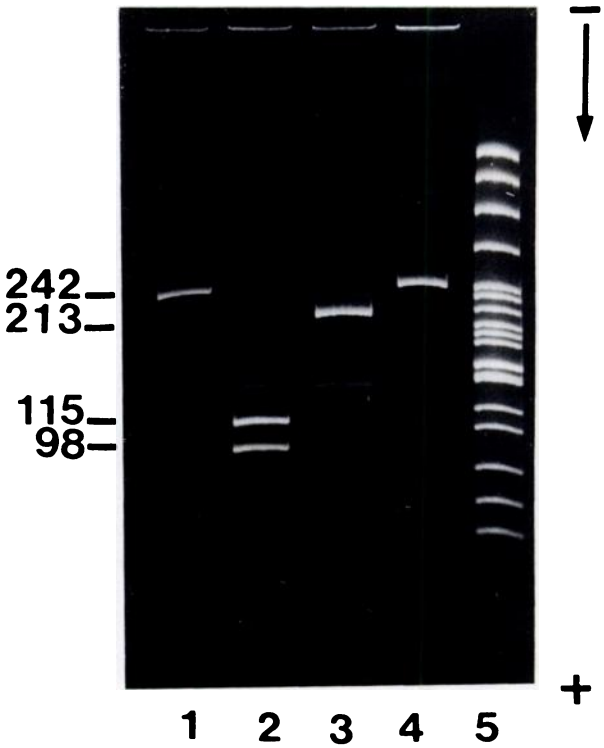

Fig 4. *Bst*NI cleavage of the 242 bp PCR fragment that includes exon 7 with the G→T mutation in nucleotide 680. Channel 1, $G6PD^{376G/680T}$, uncut; channel 2, G6PD $A^{-376G/680T}$, cut; channel 3, G6PD B, cut; channel 4, G6PD B, uncut.

Table 2. Ethnic Origin of G6PD A⁻ Subjects

Variable	376	202	680	968
Nucleotide change	A→G	G→A	G→T	T→C
Amino acid change	Asn→Asp	Val→Met	Arg→Leu	Leu→Pro
American black (n = 20)	20	18	1	1
Mexican (n = 2)	2	2		
Puerto Rican (n = 2)	2	2		
Spanish (n = 4)	4	3	1	
American white (n = 1)	1	1		
Percentage of total	100	89.7	6.9	3.4

enzyme has kinetic characteristics indistinguishable from the common G6PD A⁻ with the mutation at nucleotide 202. The difference on kinetic properties between these two apparently identical variants is unexplained. It could result from an undetected sequencing error, having missed yet a third mutation, alternative splicing producing a different protein sequence than predicted, or from an error in enzyme characterization.

Clearly the G6PD A mutation at nucleotide 376 antedated the appearance of the deficiency mutations. Only about 20% of the X-chromosomes of modern-day Africans contain the G6PD A mutation. There is no reason to suppose that the mutations found at nucleotides 202, 680, and 968 would not have been tolerated in the normal B-type enzyme, although this is a remote possibility. Given the modern gene frequency of 0.2, the probability that the second mutations occurred each time in a G6PD A individual by chance is less than 1% (ie, 0.2^3), and probably when these mutations arose G6PD A was considerably more prevalent than it is today.

When in the evolution of *homo sapiens* did the mutation at nucleotide 376 appear? Was G6PD A perhaps the "normal" genotype of primitive humans? An electrophoretically rapid G6PD exists at polymorphic frequencies in *Pan troglodytes*[17-19] and the question of whether this polymorphism is the same as that in humans has been raised.[17] We examined the nucleotide sequence of chimpanzee G6PD exon 5, which includes nucleotide 376, to determine which sequence was present in the common ancestor of these species. The results of these studies show that G6PD B was apparently the most prevalent sequence in the common precursor of *Pan troglodytes* and *Homo sapiens*: The nucleotide sequence of exon 5 of G6PD B of humans and both the rapid and slow chimpanzee G6PD are the same except for three substitutions, none of which affect the amino acid sequence (Fig 5). These findings suggest that the G6PD of early humans was G6PD B. G6PD A presumably arose in Africa, possibly after the human race had begun to disperse to Europe and Asia.[20]

Although G6PD B appears to have been the predominant type in early hominids, probably at one time in human evolution in Africa G6PD A may have been the most common genotype. This enzyme type might have provided some type of selective advantage in the African environment, or its high frequency might have occurred merely by chance. It appears to have been the predominant genotype at the time G6PD-deficient mutations became advantageous with respect to resistance against malaria,[6] explaining the fact that the deficiency mutations appear to have arisen in genes that have guanine at nucleotide 376. But as the human species evolved in Africa and selection favored the G6PD A⁻ gene, the G6PD A gene appears to have been markedly disfavored, with its frequency declining to the current level.

The mutation at nucleotide 376 is not unique to Africa. It has been detected in Spanish and Italian populations. Electrophoretically fast variants that could represent the same mutation have been noted occasionally in white populations in Brazil[21] and Cuba[22,23] and in various Middle Eastern and Asian populations.[24] However, the relative rarity of such mutations is evident from our recent examination of the RBCs of 193 phenotypically white male and female subjects, representing 305 G6PD alleles: not a single sample of G6PD A was found.

Today one can only speculate about the evolutionary forces that have shaped the shifting population frequencies of the G6PD A and G6PD B allele in humans. Possibilities may include infections such as malaria, factors that have played such a major role in selection of a variety of other genes that affect the RBC.[25]

Table 3. Biochemical Characteristics of G6PD A⁻ Variants With Mutations 202A/376G and 202A/680T

Characteristic	Mutation: 202A/376G (mean ± 1 SD, n = 8)	376G/680T Selma	376G/680T Betica
Electrophoretic mobility*			
Tris	111.0 ± 2.4	117	110
EBT	109.7 ± 3.3	114	113
Phosphate	113.6 ± 4.2	118	116
Activity (% of N)	11.6 ± 5.4	9	11.0
k_m G6P (μmol/L)	51.9 ± 9.5	40	56
k_m NADP (μmol/L)	7.0 ± 2.3	3	10
Utilization of 2-deoxy G6P (% of N)	3.0 ± 1.2	0	3.3
Utilization of deaminase NADP (% of N)	59.0 ± 9.7	86	66
Heat stability	Normal	Slightly decreased	Normal
pH optimum	Normal	Optimum at 9.5–10	Normal

G6PD Selma is from a black subject; G6PD Betica is from a Spanish subject.

*In starch gel using standard buffers,[8] expressed as percentage of normal.

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Hum G6PD B        gggctgacat ctg        tctgtgtgtc tgtctgtccg tgtctcccag
Hum G6PD A        .......... ...        .......... .......... ..........
Chimp G6PD B-like .......... ...ctgtgtg .......... .......... ..........
Chimp G6PD A-like .......... ...ctgtgtg .......... .......... ..........

Hum G6PD B        GCCACCCCAG AGGAGAAGCT CAAGCTGGAG GACTTCTTTG CCCGCAACTC
Hum G6PD A        .......... .......... .......... .......... ..........
Chimp G6PD B-like .......... .......... .......... .......... ..........
Chimp G6PD A-like .......... .......... .......... .......... ..........

Hum G6PD B        CTATGTGGCT GGCCAGTACG ATGATGCAGC CTCCTACCAG CGCCTCAACA
Hum G6PD A        .......... .......... .......... .......... ..........
Chimp G6PD B-like .......... .......... .......... .......... ..........
Chimp G6PD A-like .......... .......... .......... .......... ..........
                        ↓
Hum G6PD B        GCCACATGAA TGCCCTCCAC CTGGGGTCAC AGGCCAACCG CCTCTTCTAC
Hum G6PD A        ........G. .......... .......... .......... ..........
Chimp G6PD B-like .......... .......... ..T.....G. .......... ..........
Chimp G6PD A-like .......... .......... ..T.....G. .......... ..........

Hum G6PD B        CTGGCCTTGC CCCCGACCGT CTACGAGGCC GTCACCAAGA ACATTCACGA
Hum G6PD A        .......... .......... .......... .......... ..........
Chimp G6PD B-like .......... ....C..... .......... .......... ..........
Chimp G6PD A-like .......... ....C..... .......... .......... ..........

Hum G6PD B        GTCCTGCATG AGCCAGATgt aaggcttgcg ttgccctccc ttcccgcctg
Hum G6PD A        .......... .......... .......... .......... ..........
Chimp G6PD B-like .......... .......... .......... .......... ..........
Chimp G6PD A-like .......... .......... .......... .......... ..........

Hum G6PD B        ccaaggtggc ccaggcagtg ctc....... .......... ..........
Hum G6PD A        .......... .......... .......... .......... ..........
Chimp G6PD B-like .......... .......... .......... .......... ..........
Chimp G6PD A-like .......... .......... .......... .......... ..........
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Fig 5. Nucleotide sequence of exon 5 of the human and chimpanzee G6PD genes. Exon 5 extends from nucleotide 268 to nucleotide 485 in the consensus cDNA sequence.[14] The chimpanzee fast enzyme is designated "Chimp G6PD A-like" and the chimpanzee slow enzyme is designated "Chimp G6PD B-like." The sequence of the introns flanking exon 5 is shown in lowercase letters. Arrow points to nucleotide 376, where the A→G transition responsible for the change from human G6PD B to G6PD A is located. Dots indicate that the same base as that of human G6PD B is present.

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