The molecular basis of alkaptonuria

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Alkaptonuria (AKU) occupies a unique place in the history of human genetics because it was the first disease to be interpreted as a mendelian recessive trait by Garrod in 1902. Alkaptonuria is a rare metabolic disorder resulting from loss of homogentisate 1,2 dioxygenase (HGO) activity. Affected individuals accumulate large quantities of homogentisic acid, an intermediary product of the catabolism of tyrosine and phenylalanine, which darkens the urine and deposits in connective tissues causing a debilitating arthritis. Here we report the cloning of the human *HGO* gene and establish that it is the *AKU* gene. We show that *HGO* maps to the same location described for AKU, illustrate that *HGO* harbours missense mutations that cosegregate with the disease, and provide biochemical evidence that at least one of these missense mutations is a loss-of-function mutation.

Alkaptonuria (AKU; McKusick number 203500) is a rare autosomal recessive disorder of both historical and medical interest. It represents a classical example of a discrete biochemical lesion resulting from a single gene deficiency that gives rise to a degenerative disease[1]. AKU patients are deficient for homogentisate 1,2 dioxygenase activity (HGO, EC 1.13.11.5)[2]. This enzyme deficiency results in the accumulation of homogentisic acid (HGA), an intermediary metabolite in phenylalanine and tyrosine catabolism. In AKU patients, HGA is excreted in large amounts into the urine, which darkens on standing. This staining of the urine, which can be detected from early childhood, is the first and best known manifestation of the disease and the one that brought it to clinical attention. Over the years, benzoquinone acetic acid (an oxidation product of HGA) is deposited either directly or as a polymer into connective tissues, causing their pigmentation (ochronosis) and eventually leading to serious arthropathy[1].

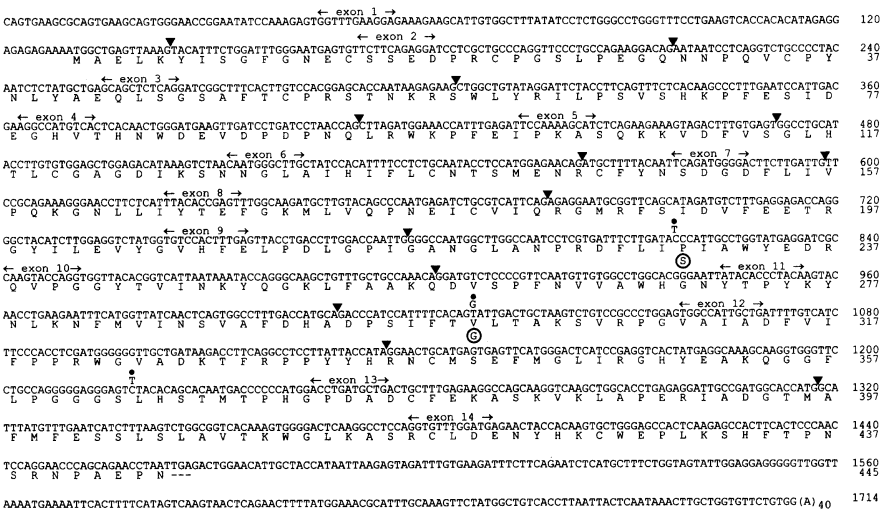
AKU was the first disease to be interpreted as a single gene trait. In 1902 Garrod reported the mode of inheritance in AKU[3]. He noted that affected individuals had normal parents and normal offspring and were frequently children of consanguineous marriages. Advised by Bateson, Garrod suggested that the peculiar form of heredity of AKU was best explained by the mendelian theories of inheritance of a recessive character[3]. In the 1908 Croonian Lectures to the Royal College of Physicians, Garrod unveiled the concept and the term *inborn error of metabolism* and went further to propose that AKU was caused by the lack of an enzyme that in normal individuals splits the aromatic ring of HGA[4]. Biochemical evidence of the defect in AKU was provided by La Du and colleagues, exactly 50 years later[2]. They demonstrated the absence of HGO activity in a liver homogenate prepared from an AKU patient and established that the defect was limited to HGO, suggesting that in affected individuals there is a failure to synthesize active enzyme[2]. In the following years no further progress in the characterization of the defect in AKU was made and it was generally accepted that AKU was caused by a defect in *HGO*. However, the fact that the *AKU* gene was not cloned precluded the molecular characterisation of this defect and indeed it was formally possible that AKU would result from a regulatory, rather than structural, gene mutation.

Major tools for the molecular characterization of the defect responsible for AKU have been developed recently. The gene responsible for AKU in humans was located to chromosome 3q2 (refs 5, 6). In addition, a mutation causing alkaptonuria has been described in mouse and mapped to chromosome 16 (ref. 7). Furthermore, a mouse homogentisate dioxygenase enzyme has been purified to homogeneity[8]. However, the decisive contribution to the characterization of the human *HGO* gene came from work with the ascomycete fungus *Aspergillus nidulans*, a model that we are currently using to study metabolic errors in human Phe catabolism[9]. In this organism, we cloned and characterized a gene encoding an HGO enzyme, denominated *hmgA*. The deduced amino acid sequence of its encoded protein product was used to identify EST (expressed sequence tag) clones putatively corresponding to the human *HGO* gene[10]. Here we demonstrate that these EST clones indeed code for human HGO and provide formal proof that AKU is due to a defect in the human *HGO* gene.

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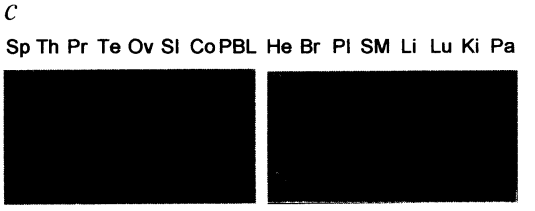


Fig. 1 cDNA nucleotide sequence, derived amino acid sequence and pattern of expression of human *HGO*. *a*, complete nucleotide sequence of the cDNA insert corresponding to EST 77725. The conceptual translation of the *HGO* ORF is shown below the cDNA sequence in one-letter code. The position of the 13 introns splitting the coding region (see text) is shown with filled triangles. Nucleotide changes found in AKU families are shown above the cDNA sequence and are indicated by black dots. Amino acid substitutions caused by two of these nucleotide changes (see text) are shown circled below the corresponding normal residue. Three dashes indicate an stop codon. *b*, Alignment of the deduced amino acid sequences of human HGO and *A. nidulans* hmgA. Identities are indicated by asterisks. *c*, Northern blot analysis of *HGO* expression in human tissues. RNA blots, prepared with 2 μg of polyadenylated mRNA from sixteen human tissues (Clontech), were hybridized with a human *HGO* cDNA clone 77725 probe. The blots were also hybridized with a human actin probe to confirm equivalent loading for each sample (not shown). Code for the RNA samples is as follows: Sp, spleen; Th, thymus; Pr, prostate; Te, testis; Ov, ovary; SI, small intestine; Co, colon; PBL, peripheral blood lymphocytes; He, heart; Br, brain; PI, placenta; SM, skeletal muscle; Li, liver; Lu, lung; Ki, kidney and Pa, pancreas.

Characterization of human *HGO* cDNA clones

The EST clones 77725 and 127783 (I.M.A.G.E. consortium, L.L.N.L), encoding putative human homologues of the fungal *hmgA* gene[10], were fully sequenced. EST-77725 contains the largest cDNA insert (1,714 bp) (Fig. 1*a*). It presents a 1,335-nt ORF encoding a 445-aa deduced polypeptide that shows 52% identity to the *A. nidulans* hmgA enzyme (Fig. 1*b*). This notable conservation in amino acid sequence indicated that the polypeptide encoded by EST-77725 is almost certainly a human protein with HGO activity. To test this possibility we expressed the polypeptide encoded by EST-77725 in *E. coli* as a GST (glutathione thiotransferase) fusion protein, and found that, in fact, it had HGO activity (see below). EST-127783 contains a 920-nt insert with a sequence identical to that of EST-77725 from codon 113 to the poly(A)$^+$ tail. From these analyses we conclude that both EST-77725 and EST-127783 are cDNA clones corresponding to the human *HGO* mRNA. We selected the EST-77725 clone for further studies because it included the entire coding region for the human HGO protein.

Tissue expression of the human *HGO* gene

It has been shown that HGO is present in human liver and kidney[2,11]. By northern blot hybridization, using the human cDNA clone 77725 as a probe, we confirmed transcription of the *HGO* gene in these tissues (Fig. 1*c*). The *HGO* probe detected a single mRNA band of approximately 1.8 kb in length, which was consistent with the length of the cDNA sequence included in clone 77725. We also detected transcription of the *HGO* gene in small intestine, colon and prostate. Steady state levels of *HGO* mRNA in the later were notably high, in agreement with previous reports describing large deposits of HGA polymers in prostates from AKU patients[1,12]. The pattern of tissue expression of the cDNA clone 77725 supports our conclusion that this EST encodes the human HGO.

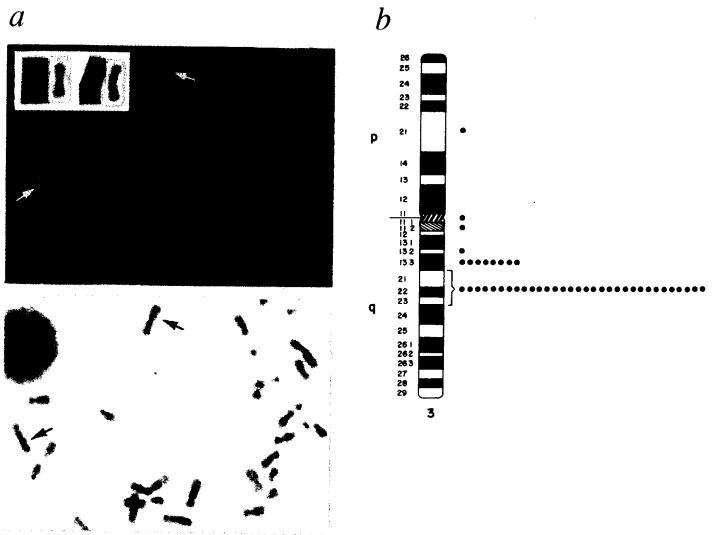


Fig. 2 Mapping of human *HGO* using FISH. *a*, Illustration of the localization of *HGO* genomic clones λAKU1 and λAKU2 to band 3q21–3q23 of DAPI-counterstained normal metaphase chromosomes (above) and illustration of the same metaphases after Giemsa banding (below). Arrows indicate specific sites of hybridization. The inset shows examples of chromosome 3 from other metaphases. *b*, Idiogram of a G-banded chromosome 3 to show the subregional distribution of chromosome 3-located fluorescent spots.

Isolation of genomic clones containing *HGO*

We used probes derived from the human *HGO* cDNA clone 77725 to screen two different EMBL-3 human genomic libraries (see Methods). We identified several overlapping clones hybridizing to the human *HGO* probe. Four of these clones, λAKU1, λAKU2, λAKU3 and λAKU4, were selected and characterized by restriction endonuclease digestion and Southern blot hybridization. λAKU1 and λAKU2 spanned a genomic region of 25 kb, approximately corresponding to the 3'-half of the human *HGO* gene, whereas clones λAKU3 and λAKU4 spanned a 30 kb-long genomic fragment, including most of the 5'- half of the gene (data not shown). The central part of the human *HGO* gene was not included in the λAKU1–4 clones. We obtained additional genomic fragments containing this portion of the gene by long-distance PCR amplification of human genomic DNA using oligonucleotide primers derived from the 3'- sequence of λAKU4, the 5'- sequence of λAKU2, and the *HGO* cDNA sequence (data not shown). The coding region of the human *HGO* gene is encoded by 14 exons spanning 60 kb of DNA. We determined the precise size of each of the human *HGO* gene exons and the sequences corresponding to their 3' and 5' intron boundaries by automatic DNA sequencing of appropriate subfragments of the λAKU genomic clones using oligonucleotide primers derived from the *HGO* cDNA sequence (Fig. 1*a*). The organization of the human *HGO* gene will be detailed elsewhere (B.G., J.M.F.-C., D.B.-V. de B., M.A.P. & S.R. de C., manuscript in preparation).

We found no evidence of multiple human *HGO* genes from the genomic screenings using the *HGO* cDNA probe. However, to rule out this possibility we performed Southern blot hybridization experiments with total human genomic DNA restricted with *Xba*I, *Pst*I, *Eco*RI or *Eco*RV. These enzymes were selected because they did not cut a 712-bp genomic fragment of the human *HGO* gene denominated G1A, which includes the entire exon 2 and 640 bp of its 5'- intronic sequence. The corresponding autoradiogram showed only one G1A-hybridizing band per digestion, indicating that *HGO* is a single copy gene in humans (data not shown).

HGO maps to human chromosome 3q21–q23

The human gene responsible for AKU has been mapped to chromosome 3q2 (refs 5, 6). Preliminary PCR experiments, using oligonucleotide primers derived from the *HGO* cDNA sequence, on a panel of hamster × human somatic cell hybrid genomic DNA samples suggested that the human *HGO* gene was also located on chromosome 3 (not shown). To confirm these data and to determine the location of the *HGO* gene in human chromosome 3, we performed fluorescent *in situ* hybridization (FISH) on chromosome preparations, using the λAKU1 and λAKU2 genomic clones (Fig. 2*a*). 63 metaphase spreads were analysed; 35 (55.5%) were found to display fluorescent spots on one or both chromatids of chromosome 3. Giemsa banding of these metaphase spreads revealed that 74% of the chromosome 3-located fluorescent spots were clustered on 3q21–q23 (Fig. 2*b*). These data demonstrate that the human *HGO* gene and the *AKU* gene map to the same chromosomal location and therefore provide cytogenetic evidence that AKU is caused by a defect in the structural gene encoding HGO.

The *HGO* gene is mutated in AKU patients

Tissue samples expressing HGO were not available from AKU patients. Thus, to establish whether *HGO* was mutated in AKU, we used genomic DNA obtained from leukocytes of all members of two unrelated Spanish AKU pedigrees (families M and S, Figs 3, 4). The parents of these families were not consanguineous. Each of the 14 exons composing the *HGO* gene were PCR-amplified and directly sequenced for each member of the two AKU families (see Methods). The data obtained demonstrated several nucleotide differences that led to the identification of different *HGO* alleles.

In family M, both parents were heterozygous at the *HGO* locus (Fig. 3). They both present a normal allele, denoted HGO^{+}, with exonic sequences that are identical to the sequence of the cloned *HGO* cDNA and a second allele that we denoted HGO^{P230S}. HGO^{P230S} contained, in addition to a 1219C→T synonymous substitution (Fig. 1*a*), two nucleotide changes that are potential loss-of-function mutations. The first was an insertion of one adenosine in intron 9, 38 bp upstream of the 3' splice site (not shown). This insertion might introduce a novel 3' splice site that, if used, would result in an almost certainly non-functional truncated polypeptide of 220 residues. The second potential loss-of-function mutation was a 817C→T transition in exon 10, changing a proline residue at position 230 to a serine residue (Fig. 1*a*). Pro230 is a conserved residue between the human and fungal proteins (Fig. 1*b*). *HGO* mutation analysis in the three offspring of family M indicated that the HGO^{P230S} allele cosegregated with the disease (Fig. 3). Thus, the two alkaptonuric children in family M, but not the healthy child, are homozygous for the HGO^{P230S} allele. The deduced product of this allele is a protein with a single amino acid substitution (Pro230Ser) as compared to the normal HGO protein.

The same 817C→T mutation, determining the

Pro230Ser substitution and the adenosine insertion in intron 9, were also found in a second alkaptonuric pedigree, family S (Fig. 4). *HGO* mutation analysis in this family showed that both parents are HGO heterozygotes. The mother is an $HGO^{+/P230S}$ heterozygote. The father has a normal allele and a novel allele that we denoted HGO^{V300G}. This novel allele is characterized by a 1028T→G transversion in exon 12, changing a valine residue at position 300 to a glycine residue (Fig. 1*a*). The deduced product of the HGO^{V300G} allele is a protein with a single amino acid substitution (Val300Gly) as compared to the normal HGO protein. Val300Gly is also conserved between the fungal and human proteins (Fig. 1*b*). Analysis of the seven offspring in this family showed cosegregation of the HGO^{P230S} and HGO^{V300G} alleles and the AKU phenotype (Fig. 4). Thus, the three alkaptonuric children in family S, but none of the four healthy children, are compound heterozygotes carrying both the 817C→T and the 1028T→G mutations.

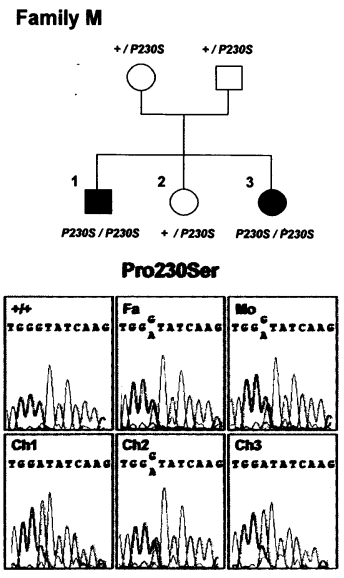


Fig. 3 Segregation of *HGO* alleles in AKU family M. Affected individuals are identified in the pedigree by solid symbols. The *HGO* alleles in each member of the family are indicated. The figure also includes the detection of the point mutation responsible for the Pro230Ser substitution in members of this family. Relevant portions of the sequencing chromatograms obtained using the reverse primer for each of the PCR-amplified genomic fragments containing *HGO* exon 10 are shown.

The analysis of the *HGO* genes in the members of our AKU families clearly demonstrated that only affected individuals inherited two *HGO* alleles with mutations that change amino acids conserved between humans and *Aspergillus*. This segregation pattern is in full agreement with the autosomal recessive mode of transmission of AKU, strongly suggesting that *HGO* is the *AKU* gene and that HGO^{P230S} and HGO^{V300G} are non-functional alleles.

The 817C→T mutation in exon 10 characterizing the HGO^{P230S} allele creates a novel *Eco*RV restriction site. We have found this to be very useful in establishing whether the Pro230Ser missense mutation was present in the normal population; individuals carrying the 817C→T mutation were easily detected by checking the appropriate amplified products for the presence of this diagnostic *Eco*RV restriction site (Fig. 5). We scanned 118 normal chromosomes from individuals of European descent; none showed the 817C→T candidate mutation. Notably, three of the four affected chromosomes in the AKU families carried the 817C→T mutation. Because it is very unlikely that this mutation is a coincidental polymorphism in AKU, we conclude that the *HGO* allele carrying the 817C→T mutation must be causative of the disorder.

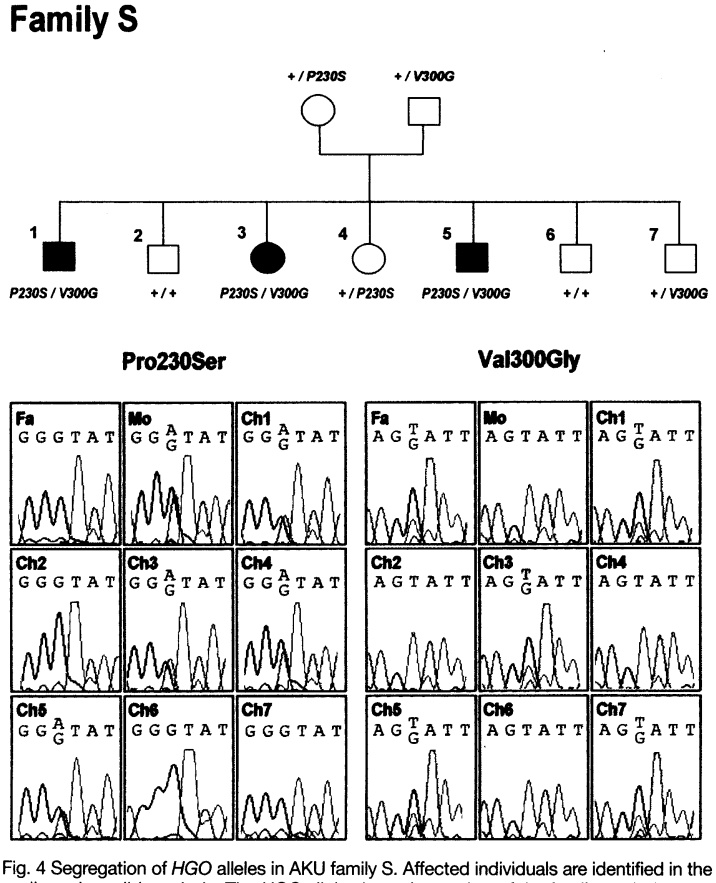


Fig. 4 Segregation of *HGO* alleles in AKU family S. Affected individuals are identified in the pedigree by solid symbols. The *HGO* alleles in each member of the family are indicated. The figure includes an illustration of the detection of the point mutations responsible for the Pro230Ser and the Val300Gly substitutions in members of family S. The figure shows relevant portions of the sequencing chromatograms of each of the PCR-amplified genomic fragments containing *HGO* exon 10 or exon 12. Exon 10 was sequenced using the reverse primer.

Pro230Ser is a loss-of-function mutation in *HGO*

We conclude from our family studies that *HGO* is the *AKU* gene, which implies that HGO^{P230S} is a non-functional allele, either because it is not properly expressed or because the encoded polypeptide lacks enzymatic activity. To establish whether the Pro230Ser missense mutation in HGO^{P230S} results in loss of enzyme activity, we constructed plasmids encoding two different GST::HGO fusion proteins, $GST::HGO^{+}$ and $GST::HGO^{P230S}$. The $GST::HGO^{P230S}$ fusion protein was identical to $GST::HGO^{+}$ except that the proline residue at HGO position 230 was changed to a serine residue. Both plasmids were transformed into *E. coli* cells and the chimaeric genes were expressed under inducing conditions. Protein extracts from these cultures and from bacteria transformed with pGEX-2T (the expression vector with no insert) were assayed for HGO activity as described[10]. We found substantial levels of HGO activity (48 nmol of maleylacetoacetate formed/min/mg of protein) in protein extracts from cultures carrying the $GST::HGO^{+}$ construct. By purification of this protein extract using glutathione-Sepharose affinity chromatography, we obtained a 27-fold increase in HGO specific activity. In contrast,



Fig. 5. Detection of the 817C→T mutation by *Eco*RV restriction analysis. Ethidium bromide staining of a 1.3% agarose gel shows PCR-amplified genomic fragments containing *HGO* exon 10 digested with *Eco*RV. *HGO* genotypes of the individuals that were included in this example are: 1, 2 and 9: $HGO^{+/+}$; 3, 4, 7, 8 and 10: $HGO^{+/P230S}$; and 5 and 6: $HGO^{P230S/P230S}$.

protein extracts from bacteria carrying the GST::HGO^{P230S} plasmid construct showed no HGO activity, either before or after the affinity purification step. We assayed two independent GST::HGO^{P230S} bacterial clones for HGO with negative results. We sequenced the HGO portion in one of the GST::HGO^{P230S} independent plasmid clones to rule out a secondary mutation introduced during the plasmid passage through *E. coli*, rather than the missense Pro230Ser mutation, as the cause of the loss of enzyme activity. Moreover, SDS-PAGE analysis of bacterial lysates containing either the GST::HGO^{+} or the GST::HGO^{P230S} plasmid construct showed that both proteins were induced to a similar extent, confirming the integrity of both reading frames and indicating similar stability for the two polypeptides. As a whole, our results from the analysis of the GST::HGO fusion proteins indicate that the single amino acid substitution Pro230Ser in human HGO is sufficient to determine loss of enzyme activity. Therefore we provide biochemical evidence that at least one of the missense mutations found homozygously in the *HGO* gene in AKU patients is a loss-of-function mutation, establishing that *HGO* is the *AKU* gene.

Discussion

We characterized a human cDNA clone encoding a protein showing 52% identity in deduced amino acid sequence to *A. nidulans* HGO. Because this human protein has HGO activity when expressed in *E. coli* we conclude that the gene corresponding to this cDNA is the human *HGO* gene. The results of our FISH analysis indicate that the map position of *HGO* coincides with the *AKU* locus, strongly suggesting that *HGO* is the *AKU* gene. Furthermore, we found loss-of-function mutations in *HGO* in AKU patients. In summary, we provide experimental data to formally establish that AKU is due to a defect in this *HGO* gene.

AKU is a rare disorder with an incidence in the general population of 1 in 250,000 individuals. It was, therefore, fortunate to collect two complete AKU pedigrees including 14 individuals and five affected children to perform the *HGO* mutation analysis. We emphasize that there is no blood relation between the two families, which are not consanguineous. We showed that both parents in these two families are heterozygotes for a normal *HGO* allele and a mutated *HGO* allele, and only those offspring who inherit the paternal and maternal mutated *HGO* alleles are affected by AKU (Figs 3, 4). The two mutated *HGO* alleles identified in the two AKU families were denominated HGO^{P230S} and HGO^{V300G}. They both present missense mutations changing amino acid residues that are conserved between the human and fungal HGO proteins. Using an *E. coli* expression system, we demonstrated that the Pro230Ser amino acid substitution characteristic of the HGO^{P230S} allele is sufficient to cause the loss of enzyme activity — thus HGO^{P230S} is a non-functional allele. As a whole, these results provide experimental evidence that children 1 and 3 in family M (Fig. 3), who are HGO^{P230S} homozygotes, are alkaptonuric because they cannot synthesize active HGO. Based on the *HGO* segregation data in family S, we anticipate that HGO^{V300G} is also a non-functional allele.

AKU is considered a relatively benign disorder and affected individuals have a normal life expectancy[13,14]. AKU patients suffer, however, from debilitating arthritis, whose effects tend to aggravate with age. Although the molecular basis of alkaptonuria is established here, the mechanism by which this genetic defect leads to the eventual development of degenerative arthritis of the spine and large peripheral joints remains to be identified. It is generally accepted that damage to connective tissues in joints results from deposition in these tissues of ochronotic pigment. This pigment probably results from oxidation of HGA to benzoquinone acetic acid which is converted to a polymerized product. A polyphenol oxidase, the enzyme system mediating this conversion, has been identified in skin and cartilage[15]. Treatment with ascorbic acid, which causes a marked inhibition of this enzyme reaction[15], is followed by disappearance of benzoquinone acetate from urine without affecting excretion of homogentisate[16]. It has therefore been proposed that ascorbic acid treatment could prevent development of ochronotic arthritis[16]. Mice endogenously synthesize ascorbic acid[17]. Alkaptonuric mice, homozygous for the *Aku* mutation, do not show any pigmentation of connective tissues and do not develop arthritic lesions[18] (showing high urinary excretion of homogentisic acid), supporting the view that ascorbic acid treatment would prevent ochronotic arthritis[7,18]. However, mice might be lacking polyphenol oxidase or, alternatively, the homozygous *Aku* mutation could have resulted in the loss of both homogentisate dioxygenase and polyphenol oxidase. The cloning of the mouse *HGO* gene will permit testing this possibility by targeted disruption.

Black calculi in prostate are common in male AKU patients, who might occasionally require prostatectomy. It has been suggested that alkaline secretions in this organ promote rapid polymerisation of HGA and the resulting pigment then would afford a nidus for stone formation[1]. One unexpected result of our work was the finding of high levels of *HGO* mRNA in prostate. This suggests that catabolism of phenylalanine plays an active role in the physiology of this organ and would provide an alternative explanation for the above abnormalities. This finding also raises questions concerning whether normal prostatic functions are altered in AKU.

Attempts to identify heterozygote carriers by detection of HGA urinary excretion after loading with tyrosine have been unsuccessful[19]. The molecular cloning of the *HGO* gene makes heterozygote detection possible. The presence of the 817C→T mutation in three of the four affected chromosomes in the AKU families studied here raises the question of whether the Pro230Ser substitution is the predominant mutation in AKU or is perhaps distinctive of the Spanish population. This and other issues concerning the genetic polymorphism at the *HGO* locus will be addressed in future studies.

Table 1 PCR primers for genomic amplification of the human *HGO* exons (5'→3')

Exon	Forward	Reverse	PCR product (bp)
1	GGGAATTCTGTAACCGGTTAAATAAGCTTG	CTGGATCCCTGAAGTTCTCAGAAACTTCC	316
2	CATATAGGAATTCTTACCTTAGTAAACTGC	GGCAGATCTTTGGCCTGAAAGCTAGTCATCC	242
3	CTGAATTCGTTGGTGGGAAGGTGGGATGC	CTGTGGATCCCAGGGGGAAGGAGGCAGCAC	249
4	GTATCATTGCTTCACTGCTTC	TTCCAATGACCATGGTATTC	338
5	GATGCTATTTATTTAGAAGGTC	GCTTTTGCTTGGCATTCAGGCTGCAAATGG	225
6	GGATTCCAAACGTCCCACCGGTCCC	CAACTATGTATGTGCATATGTGAC	338
7	CAATGATTTGTGTTATGACG	GAAATAAGACAGATTGAAGAATGAAGG	223
8	GGAATTCAAAAGCAAATGCAGCCTTAAGCC	GTTGGATCCAGCAGCAGCTGAAACATCTG	279
9	CGGGCTATGGATCCGGGCAGCTTCTTTATAAC	GTGGAATTCTGAGTGAGACAGCGAAGGGAG	317
10	GTGGATCCGCTCTTGACATGAAAG	GCTGAATTCGTAGTCCCGTAGTGG	413
11	CAGAATTCTACTTCTCCCAAAGG	CTGTGGATCCCTCCCACCCAAGCG	358
12	GGAATTCACTTGCAAACACTTCATGTATGC	CCAGGATCCTTCCCAATGTGTAGCGCTCAC	340
13	GGGGATCCGTGTTTTACTGGTCTTGCCTTG	GGAATTCCTCTGTGACTTTGGAG	346
14	GGAATTCTTATTGGAAAATTTCACCTACCC	GAAGGGATCCAGGCCAACCACAAGGC	589

In some cases, the *HGO* sequence has been modified to introduce *Eco*RI, *Bam*HI or *Bgl*II restriction sites (underlined) for subcloning of the amplified product.

Methods

Library screening. Two human genomic libraries constructed in the λEMBL-3 vector (HL-1006d and HL-1067j; Clontech) were screened with (^{32}P) labelled probes derived from the human EST-77725 cDNA clone. λEMBL-3 clones were isolated by standard techniques, and restriction fragments were subcloned into pBluescript SK$^+$ (Stratagene).

Samples. Affected individuals in families M and S were diagnosed with alkaptonuria based on routine childhood analyses for homogentisic aciduria. DNA samples for all members of families M and S, and for normal individuals were obtained from peripheral blood lymphocytes using standard methods. Blood was collected from patients and their relatives following informed consent.

PCR amplification of *HGO* exons and sequencing. Exons of the HGO gene were amplified from genomic DNA using specific primers derived from the 5' and 3' intronic sequences, and the corresponding PCR products were purified by agarose gel electrophoresis. Direct sequencing of PCR products, as well as double stranded sequencing of plasmid inserts, was performed automatically in an ABI-377 sequencer using FS *Taq* polymerase and dye terminators. Sequences of oligonucleotides used for PCR amplification and sequencing of *HGO* exons are shown in Table 1. Annealing temperature for all primer pairs was 60 °C. In some cases, we modified the original intronic sequences by introducing restriction sites to facilitate the cloning of the amplified products. The sequence of the complete EST-77725 cDNA encoding human *HGO* has been submitted to the GenBank/EMBL Data Bank (accession number: U63008).

***In situ* hybridization.** The human genomic clones λAKU1 and λAKU2 were labelled by nick translation with digoxigenin-11-dUTP. FISH was carried out as described[20] on chromosome preparations obtained from phytohaemaglutinin-stimulated human lymphocytes. Hybridization was done in 50% deionized formamide, 10% dextran sulphate, 2× SSC, 50 mM phosphate pH 7, 200 ng/ml of yeast tRNA, 200 ng/ml of salmon sperm DNA, 4 ng/ml of labelled probe and 100-fold excess of both sonicated human placental DNA and Cot-1 DNA. Preannealing of the probe-competitor mixture was done for 1 h at 37 °C and the chromosome preparations hybridized overnight at 37 °C. Stringency washes were done at 45 °C in 2× SSC containing 50% formamide. Immunochemical detection was performed with (TRITC)-labelled anti-digoxigenin antibodies. G-banding analysis was done after FISH, fading the slides in 70% and 95% ethanol and staining with 0.15% (w/v) Leishman in methanol, diluted 1:3 in 25 mM phosphate pH 6.8.

GenBank/EMBL accession number. *H60* (EST 77725): U63008.

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