Identification of Mutations Associated with Peroxisome-to-Mitochondrion Mistargeting of Alanine/Glyoxylate Aminotransferase in Primary Hyperoxaluria Type 1

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Abstract. We have previously shown that in some patients with primary hyperoxaluria type 1 (PH1), disease is associated with mistargeting of the normally peroxisomal enzyme alanine/glyoxylate aminotransferase (AGT) to mitochondria (Danpure, C. J., P. J. Cooper, P. J. Wise, and P. R. Jennings. *J. Cell Biol.* 108:1345–1352). We have synthesized, amplified, cloned, and sequenced AGT cDNA from a PH1 patient with mitochondrial AGT (mAGT). This identified three point mutations that cause amino acid substitutions in the predicted AGT protein sequence. Using PCR and allele-specific oligonucleotide hybridization, a range of PH1 patients and controls were screened for these mutations. This revealed that all eight PH1 patients with mAGT carried at least one allele with the same three mutations. Two were homozygous for this allele and six were heterozygous. In at least three of the heterozygotes, it appeared that only the mutant allele was expressed. All three mutations were absent from PH1 patients lacking mAGT. One mutation encoding a Gly→Arg substitution at residue 170 was not found in any of the control individuals. However, the other two mutations, encoding Pro→Leu and Ile→Met substitutions at residues 11 and 340, respectively, cosegregated in the normal population at an allelic frequency of 5–10%. In an individual homozygous for this allele (substitutions at residues 11 and 340) only a small proportion of AGT appeared to be rerouted to mitochondria. It is suggested that the substitution at residue 11 generates an amphiphilic alpha-helix with characteristics similar to recognized mitochondrial targeting sequences, the full functional expression of which is dependent upon coexpression of the substitution at residue 170, which may induce defective peroxisomal import.

PRIMARY hyperoxaluria type 1 (PH1)[1] is a lethal autosomal recessive disease caused by a deficiency of the liver-specific peroxisomal enzyme alanine/glyoxylate aminotransferase 1 (AGT, EC 2.6.1.44) (Danpure and Jennings, 1986). Whereas most PH1 patients have a complete deficiency of AGT enzyme activity (Danpure and Jennings, 1988) and AGT immunoreactive protein (Wise et al., 1987), about one-third possess significant amounts of residual AGT activity and protein. In all of these latter patients so far examined it appears that the disease is due, at least in part, to a unique protein targeting defect in which AGT is erroneously routed to the mitochondrion instead of its normal intracellular location, the peroxisome (Danpure et al., 1989, 1990).

The subcellular distribution of hepatic AGT is species specific. In cat and dog AGT is mitochondrial, whereas in rabbit, guinea pig, macaque, and human the enzyme is confined to the peroxisome (Noguchi and Takada, 1979; Takada and Noguchi, 1982*a*,*b*; Okuno et al., 1979). In contrast, rat, mouse, and hamster have significant levels of AGT present in both peroxisomes and mitochondria (Noguchi et al., 1979; Takada and Noguchi, 1982*a*,*b*). In an attempt to explain the molecular basis of the rerouting phenomenon in PH1 patients with mitochondrial AGT (mAGT), we recently determined the nucleotide sequence of normal human peroxisomal AGT (pAGT) cDNA (Takada et al., 1990), and compared this with the published cDNA sequence encoding rat mAGT (Oda et al., 1987). This revealed that the human cDNA has a sequence identity with its counterpart in the rat of 74% within the region encoding the rat amino-terminal mitochondrial targeting sequence (MTS), but that this MTS is not expressed as part of the human protein due to a coding difference at the site corresponding to the rat protein translation start site (ATG in the rat gene, ATA in the human gene). This provided an explanation for the exclusive peroxisomal localization of AGT in human liver. In addition, the high se-

1. *Abbreviations used in this paper*: AGT, alanine/glyoxylate aminotransferase 1; ASO, allele-specific oligonucleotide; mAGT, mitochondrial AGT; MTS, mitochondrial targeting sequence; pAGT, peroxisomal AGT; PCR, polymerase chain reaction; PH1, primary hyperoxaluria type 1; PTS, peroxisomal targeting sequence.

quence identity between the sequences in this region (74% compared to 79% between the shared coding regions) opened up the possibility that mutations which extend the open reading frame of the human gene to include additional amino-terminal sequences corresponding to partial or complete versions of the rat MTS might be a factor in the rerouting of AGT in PHI.

In the present study we have determined the AGT cDNA sequence of a PHI patient with mAGT, and screened genomic DNA from a range of PHI and normal individuals for the differences observed between this sequence and that of normal human AGT cDNA. We discuss the possible role of these mutations in the rerouting of AGT to the mitochondria.

Materials and Methods

Isolation of poly-A$^+$ RNA

Total RNA was isolated from liver stored in liquid nitrogen by the guanidinium isothiocyanate/caesium chloride method (Chirgwin et al., 1979), and poly A$^+$ mRNA was selected by oligo dT-cellulose (Boehringer Mannheim UK, Lewes, East Sussex, UK) column chromatography (Aviv and Leder, 1972).

Synthesis, Polymerase Chain Reaction Amplification, and Cloning of cDNA

For cDNA synthesis, 2 μg of poly-A$^+$ RNA was heated to 90°C for 5 min, chilled on ice, and incubated with 500 U of M-MLV reverse transcriptase (Gibco BRL, Paisley, Scotland, UK) and 50 pmol of primer in a total volume of 25 μl of reverse transcription buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 100 μg/ml BSA, 1 U/μl placental ribonuclease inhibitor (Boehringer Mannheim UK, Lewes, East Sussex, UK) for 10 min at room temperature, and then for 90 min at 42°C. Total reverse transcription reactions (25 μl) were added to 75 μl of polymerase chain reaction (PCR) buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% [wt/vol] gelatin) containing an additional 50 pmol of the reverse transcription primer and 100 pmol of a second PCR primer. Cloned Taq Polymerase (2.5 U) (Amplitaq; Perkin Elmer Corp., Norwalk, CT) was added, and the reactions were thermally cycled (94°C for 90 s, 55°C for 90 s, 72°C for 60 s, for 35 cycles, and then 72°C for 10 min) under mineral oil. PCR products were identified by agarose gel electrophoresis and Southern blotting, gel purified, and cloned into pUC19. The sequences of the primers for reverse transcription/PCR are given in the Oligonucleotide subsection.

Purification and Sequencing of Plasmid DNA

Plasmid DNA was prepared by the alkaline/SDS lysis method (Maniatis et al., 1982) from 5 ml of log-phase culture, and sequenced using modified T7 DNA Polymerase (Sequenase; United States Biochemical Corporation, Cleveland, Ohio). In some instances plasmid DNA from 250-ml cultures was purified on caesium chloride gradients before sequencing. *E. coli* strain JM101 was used throughout.

Isolation and Characterization of a Genomic Clone Encoding AGT

Approximately 1 × 10^6 plaque forming units of a library of 10–20 kbp Sau3A partial digestion products of human DNA, cloned into the vector lambda 47.1 (Loenen and Brammar, 1980) were screened by plaque hybridization (Benton and Davis, 1977). The probe was full-length normal AGT cDNA, labeled by random priming (Feinberg and Vogelstein, 1984). A single hybridization positive was plaque purified, and DNA was prepared from plate lysates of this clone (L-AGTI) (Helms et al., 1985) and restriction mapped. Regions of this clone with homology to specific regions of the cDNA were subcloned into pUC19 and sequenced.

Isolation and PCR Amplification of Blood Genomic DNA

High molecular weight genomic DNA was isolated from whole blood by standard techniques. 1-μg aliquots of purified DNA were heated at 95°C for 5 min before addition to the PCR reaction (100 μl). PCR conditions were as described above. The sequences of the PCR primers are given in the Oligonucleotide subsection.

Allele-specific Oligonucleotide Hybridization

PCR product (5 μl/spot) was denatured in 0.3 M NaOH, neutralized in 0.3 M Tris-HCl, pH 7.4/0.75 M NaCl, spotted onto nylon membrane (Hybond-N; Amersham International plc, Amersham, Bucks., UK) and UV-fixed. Oligonucleotide probes (20 pmol) were end-labeled (Maniatis et al., 1982) and purified on Nensorb columns (DuPont UK, Stevenage, Herts., UK). Prehybridization (>4 h) and hybridization (>16 h) were at 37°C in 0.1% (wt/vol) SDS, 0.2% (wt/vol) BSA, 0.2% (wt/vol) Ficoll, 0.2% (wt/vol) polyvinyl pyrrolidone, 100 μg/ml boiled, sonicated herring sperm DNA, and 6× SSC (1× SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0). The filters were washed at 37°C in 6× SSC, 0.1% (wt/vol) SDS (15 min, twice), then in 3 M tetramethylammonium chloride, 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.1% (wt/vol) SDS (3 × 15 min, once at 37°C, then twice at 47°C) (Wood et al., 1981).

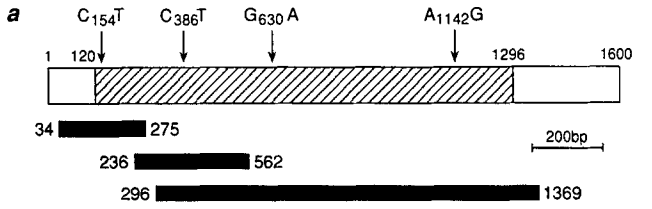
Oligonucleotides

All oligonucleotides were synthesized using a Milligen/Biosearch 7500 DNA synthesizer (Millipore (UK) Ltd., Watford, Hertfordshire, UK). Oligonucleotides used as allele-specific probes are described in the legend to Fig. 5. For amplification of cDNA by reverse transcription/PCR three pairs of primers (PA/PB, PC/PD, PE/PF) were used. PA, PC, and PE were antisense (reverse transcription) primers. PB, PD, and PF were the respective primers for synthesis of second strand cDNA. The sequences of these primers were as follows, with the corresponding positions relative to full-length AGT cDNA shown in parentheses. PA, 5′-TCCATGATCTGGTACATATC-3′ (nucleotides 295–276); PB, 5′-CACCAATCCTCACCTCTCAC-3′ (14–33); PC, 5′-TTAAGAACAGCAGCACTGGC-3′ (582–563); PD, 5′-AACCTGCCTCCTCGCATCAT-3′ (216–235); PE, 5′-GGACCTTGCAGGGTCTGTTT-3′ (1,389–1,370); PF, 5′-GATATGTACCAGATCATGGA-3′ (276–295). PCR primers for amplification from genomic DNA were based upon sequences from the AGT genomic clone, the full structure of which will be published elsewhere (Purdue, P. E., M. Lumb, M. Fox, G. Griffo, C. Hamon-Benais, S. Povey, and C. J. Danpure, manuscript submitted for publication). The sequences of the three pairs of primers (PB/PG, PH/PI, PJ/PK) are as follows. PB, see above; PG, 5′-ATCTGTGGGTGGGAACATGG-3′; PH, 5′-GCCAGTGCTGCTGTTCTTAA-3′; PI, 5′-TCTGAGCTGAGCTCCAGTCC-3′; PJ, 5′-TTCCCACAGTCACCACTGTG-3′; PK, 5′-CCTGGTGCACAGTCCTGCTC-3′.

Results

cDNA Sequence Analysis

Three overlapping regions of cDNA covering nucleotides 34–1,369 (relative to the 5′ end of the full-length cDNA; Takada et al., 1990), were synthesized from poly A$^+$ RNA extracted from the liver of a PHI patient with mAGT (patient A), and amplified by PCR (Fig. 1 *a*). These cDNA products were cloned and sequenced, revealing a total of five differences to the normal cDNA sequence. (Throughout this paper X_nY is used to denote an X to Y mutation at nucleotide n relative to the 5′ end of full-length AGT cDNA.) One of these, $T_{937}A$, was identified as being an artefact of the PCR process by the following criteria. First, this change was only present in a single clone out of three sequenced in this region, despite evidence (discussed later) that all the AGT mRNA derives from a single allele in this patient, and secondly, the loss of a site for the restriction enzyme Dde I predicted by this mutation could not be detected when total PCR product was digested by this enzyme and analyzed by



b

Nucleotides (Nucl.)	Amino Acids (AA)	Nucl. sequence & predicted AA sequence: Normal			Patient A		
150-158	10-12	CCC	CCC	AAG	CCC	CTC	AAG
		P	P	K	P	L	K
627-635	169-171	TTC	GGG	GAA	TTC	AGG	GAA
		F	G	E	F	R	E
1137-1145	339-341	GTC	ATA	GAC	GTC	ATG	GAC
		V	I	D	V	M	D

c

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 1 MASHKLLVTPLKALLKPLSIPNQLLLGPGPSNLPPRIMAAGGLQMIGSMSKDMYQIMDEI 60
                P

61 KEGIQYVFQTRNPLTLVISGSGHCALEAALVNVLEPGDSFLVGANGIWGQRAVDIGERIG 120

121 ARVHPMTKDPGGHYTLQEVEEGLAQHKPVLLFLTHGESSTGVLQPLDGFRELCHRYKCLL 180
                                              G

181 LVDSVASLGGTPLYMDRQGIDILYSGSQKALNAPPGTSLISFSDKAKKKMYSRKTKPFSF 240

241 YLDIKWLANFWGCDDQPRMYHHTIPVISLYSLRESLALIAEQGLENSWRQHREAAAYLHG 300

301 RLQALGLQLFVKDPTLRLPTVTTVAVPAGYDWRDIVSYVMDHFDIEIMGGLGPSTGKVLR 360
                                    I

361 IGLLGCNATRENVDRVTEALRAALQHCPKKKL 392
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Figure 1. Analysis of AGT cDNA from a patient with mAGT (patient A). (*a*) cDNA (nucleotides 34–1,369, excluding primers) was synthesized in three overlapping segments which are shown as filled bars aligned with a representation of full-length AGT cDNA (*open bar*, with the coding region hatched). Numbers are base pairs relative to the 5′ end of the full-length cDNA (Takada et al., 1990) and correspond to the 3′ ends of the PCR primers. The positions of the identified mutations are shown with arrows. (*b*) The predicted amino acid changes resulting from the mutations at positions 154, 630, and 1,142. Amino acid residues are numbered relative to the amino-terminal methionine. (*c*) The derived amino acid sequence of AGT from patient A. The positions of the differences to the normal sequence are shown in bold, with the corresponding amino acid in the normal sequence shown below. These sequence data are available from EMBL/GenBank/DDBJ under accession number X53414.

agarose gel electrophoresis (not shown). The remaining four observed differences were interpreted as being genuine mutations, being present in every clone (minimum three) sequenced. Of these four point mutations, one ($C_{386}T$) is in the wobble position of an alanine codon, but the other three result in alterations in the predicted protein sequence, as shown in Fig. 1, *b* and *c*.

Isolation and Characterization of a Genomic Clone Encoding AGT

For the majority of individuals, both with PH1 and controls, whom we wished to screen for the identified mutations, it was not possible to synthesize cDNA due to the nonavailability of suitable liver tissue. As a prerequisite to direct analysis of AGT sequences within genomic DNA isolated from the blood of these individuals, a genomic clone encoding normal human AGT was isolated and partially characterized. A partial restriction map of this clone is shown in Fig. 2. Regions of the clone that correspond to the mutant sites within the cDNA were identified by Southern blotting (not shown). These were a 1.42-kbp Sph I–Sph I fragment with homology to a probe covering nucleotides 1–250 of the cDNA, a 2.0-kbp Sph I–Bam HI fragment with homology to a probe covering nucleotides 535–615 of the cDNA, and a 0.9-kbp Pst I-Pst I fragment that hybridized to a probe of nucleotides 1,050–1,270 of the cDNA. These fragments were cloned into pUC19 and sequenced. As shown in Fig. 2, in each case these clones span the entire exon carrying the mutant site. Clone pAX covers the first exon (cDNA nucleotides 1–287) together with ~1.0 kbp of 5′ nontranscribed DNA and 135 bp of the first intron. Clone pAY includes an exon of 101 bp (cDNA nucleotides 546–646) and flanking intron sequences, and clone pAZ includes an exon of 129 bp (cDNA nucleotides 1,065–1,193) with flanking intron sequence and part of the next exon. Using PCR primers based on the sequences of these clones, products of 328, 126, and 193 bp, which include the sites of mutations $C_{154}T$, $G_{630}A$, and $A_{1142}G$, respectively, were amplified from DNA extracted from the blood of individuals A–L and a number of controls whose subcellular distribution of AGT is unknown.

The $C_{154}T$ Mutation Is a Common Feature of PH1 Patients with mAGT

Of the three mutations identified in the cDNA of patient A,

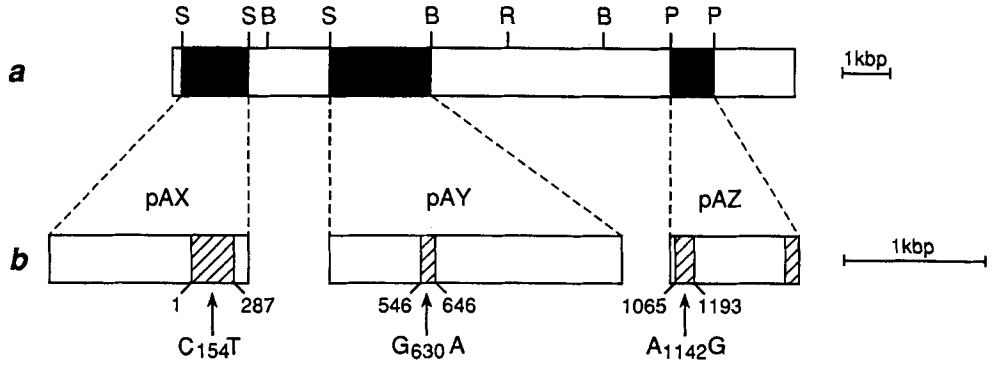

Figure 2. Partial physical map of genomic clone L-AGT1. (*a*) The filled boxes represent regions that hybridized to probes corresponding to specific regions of AGT cDNA, as described in the text. These fragments were subcloned into pUC19 to produce clones pAX, pAY, and pAZ. *P*, Pst I; *B*, Bam HI; *S*, Sph I; *R*, Eco RI. (*b*) The structure of clones pAX, pAY, and pAZ as deduced from their sequences. Exons are represented with hatched boxes and introns with open boxes. The numbers represent the positions of the cDNA sequence corresponding to the intron/exon boundaries. The mutation sites are marked with arrows.

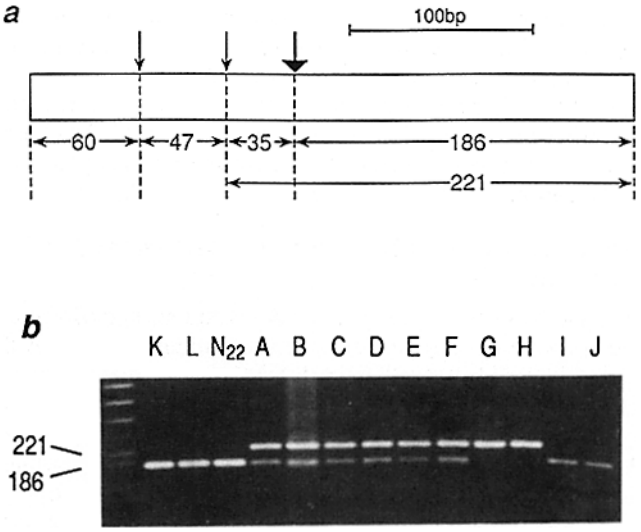

Figure 3. StyI digestion of exon 1 PCR product from eleven PH1 patients. (*a*) Sty I sites within exon 1 PCR product. The $C_{154}T$ mutation destroys a site for the restriction enzyme Sty I (CCAAGG mutated to TCAAGG). Whereas normal exon 1 PCR product (328 bp) will give Sty I digestion products of 47, 60, 35, and 186 bp, the PCR product from a $C_{154}T$ mutant allele will digest to products of 47, 60, and 221 bp. Sty I sites are marked with arrows, with the polymorphic site indicated by the broad arrowhead. (*b*) Ethidium bromide stained 2% agarose gel of Sty I digestion products from PH1 patients (*A–K*) and controls (*L*, N_{22}). The sizes are in base pairs. The molecular weight markers were the 1-kbp ladder (Gibco BRL, Paisley, Scotland, UK). The reason for the lower intensity of the 186-bp band relative to the 221-bp band in patients *A–F* is unclear, but is possibly due to formation of heteroduplexes, which would not be cleavable at the polymorphic site, during the later stages of the PCR.

the $C_{154}T$ mutation seemed the most likely to have a direct causal role in the generation of a MTS, for the following reasons. First, it is to be expected that the signals responsible for targeting of the mutant AGT to the mitochondria will, in common with the MTSs of the vast majority of imported mitochondrial proteins, be expressed as an amphiphilic amino-terminal domain (Schatz, 1987; Roise and Schatz, 1988). Whereas it is not easy to envisage the $G_{630}A$ or $A_{1142}G$ mutations directly affecting the amino-terminal structure of AGT, it seems entirely possible that the proline to leucine conversion at residue 11 caused by the $C_{154}T$ mutation could induce significant structural alterations in this region. Secondly, the amino-terminal sequence of AGT displays a number of the features common to MTSs (e.g., high in basic amino acids, no acidic amino acids), supporting the argument that structural alterations in this region could result in formation of a peptide domain with mitochondrial targeting ability.

For these reasons individuals A–L were initially screened for this mutation. As shown in Fig. 3 *a*, the $C_{154}T$ mutation destroys a site for the restriction enzyme Sty I, resulting in the generation of a novel 221-bp Sty I–Sty I restriction fragment. In the absence of the $C_{154}T$ mutation, this region is digested to products of 186 and 35 bp. First exon PCR product from PH1 patients A–K and two controls (L,N_{22}) were digested with Sty I and fractionated by agarose gel electrophoresis (Fig. 3 *b*). While the two controls and the three PH1 patients without mAGT gave a digestion product of 186 bp (but no product of 221 bp), suggesting that these individuals are homozygous for the normal allele, the digestion products from all eight PH1 patients with mAGT include a fragment of 221 bp, implying the presence of an allele carrying the $C_{154}T$ mutation. Patients A–F, whose AGT activities range from 0–12.9% of normal (Table I), appear to be heterozygous for the mutation, in that they also have a 186-bp digestion product, while patients G and H, whose AGT activities are 27.1 and 28.7% of normal, respectively (Table I), have no 186-bp product, implying that they are homozygous for the $C_{154}T$ mutation.

Three Patients Heterozygous for the $C_{154}T$ Mutation Express Only the Mutant Allele

Liver tissue suitable for extraction of intact RNA was available from three (A–C) of the six patients heterozygous for the $C_{154}T$ mutation. mRNA was prepared from these livers and from the liver of patient K (normal level of pAGT protein, but a complete deficiency of AGT enzyme activity) and a normal control (X, AGT distribution unknown), and cDNA encoding nucleotides 14–295 (including flanking primer-derived sequence) was synthesized and amplified as described earlier. As for the PCR products from genomic DNA, the $C_{154}T$ mutation is detectable by virtue of the Sty I polymorphism, giving a Sty I digestion product of 175 bp from PCR product derived from mutant cDNA, compared to 140 bp for the normal cDNA (Fig. 4 *a*). The Sty I digests of the PCR product from patients A, B, C, K and the normal control (X) were fractionated by agarose gel electrophoresis, Southern

Table I. Description of Patients

Patient	AGT	CRM	Subcell	Mutations		
				$C_{154}T$	$G_{630}A$	$A_{1142}G$
	%					
A	8.7	+	M	C/T	G/A	A/G
B	8.2	+	M	C/T	G/A	A/G
C	5.6	+	M	C/T	G/A	A/G
D	12.9	+	M	C/T	G/A	A/G
E	7.8	+	M	C/T	G/A	A/G
F	0.0	+	M	C/T	G/A	A/G
G	27.1	++	M	T/T	A/A	G/G
H	28.7	++	M	T/T	A/A	G/G
I	0.0	−	−	C/C	G/G	A/A
J	0.7	−	−	C/C	G/G	A/A
K	1.8	+++	P	C/C	G/G	A/A
L	106.2	+++	P	C/C	G/G	A/A
M	92.1	+++	P/M	T/T	G/G	G/G

Patients *A–K* have PH1, whereas patients *L* and *M* have idiopathic oxalosis of unknown etiology (possibly secondary to renal failure in *L* and oxalate hyperabsorption in *M*). *AGT*, enzyme activity, corrected for crossover from glutamate/glyoxylate aminotransferase, expressed as percent of mean control level (Danpure and Jennings, 1988). *CRM*, estimated level of immunoreactive AGT protein (+++, normal; ++, intermediate; +, low; −, undetectable) as determined by immunoblotting (Wise et al., 1987). *Subcell*, subcellular distribution of immunoreactive AGT protein (*M*, > 90% mitochondrial, *P*, 100% peroxisomal, *P/M*, ~90% peroxisomal, ~10% mitochondrial) as determined by immunoelectron microscopy (Cooper et al., 1988). *Mutations*, sequence at the mutant sites identified in this study. $C_{154}T$, $G_{630}A$, and $A_{1142}G$ are the three mutations for which the patients were screened. *A*, *C*, *G*, and *T*, the bases present in each allele at the mutant site.

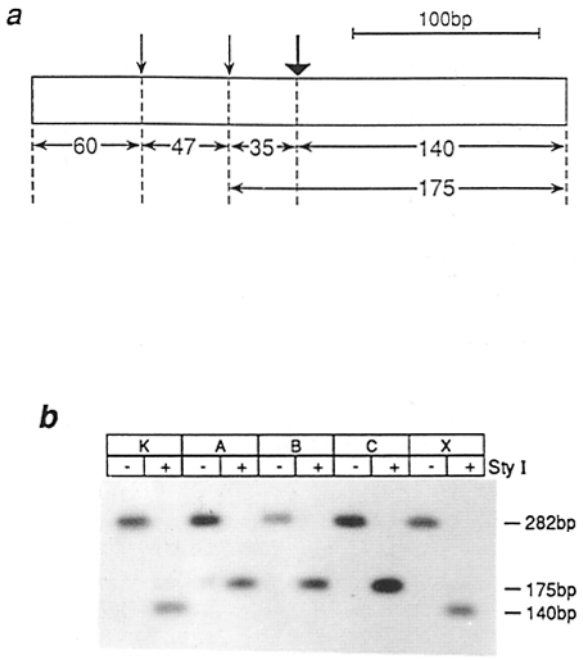

Figure 4. Southern blot of Sty I digestion products of cDNA from four PHI patients. (*a*) Sty I sites within the normal and $C_{154}T$ mutant AGT cDNA (nucleotides 14–295). Normal cDNA gives restriction digestion products of 47, 60, 35, and 140 bp, and the mutant cDNA gives products of 47, 60, and 175 bp. Sty I sites are marked with arrows, with the polymorphic site indicated by the broad arrowhead. (*b*) Southern blot of AGT cDNA (nucleotides 14–295, including 20 bp of primer sequence at either end), from four PHI patients (*A*, *B*, *C*, *K*) and a control (*X*), fractionated through a 2% agarose gel before (−) and after (+) Sty I digestion. The probe was normal AGT cDNA (nucleotides 14–295). Patients *A–C* have mAGT, patient *K* has pAGT immunoreactive protein, and control *X* has unknown AGT distribution.

blotted, and probed with AGT cDNA. This blot (Fig. 4 *b*) revealed that, whereas patient K and the normal control gave a single band at 140 bp, the patients heterozygous for this mutation (A–C) gave a single band of 175 bp. This implies that all the cDNA synthesized in patients A–C contains the $C_{154}T$ mutation, which in turn suggests that the only allele expressed in the livers of these patients is the mutant one. In the case of patient A this is consistent with the finding that all cDNA clones sequenced contained the $C_{154}T$, $C_{386}T$, $G_{630}A$, and $A_{1142}G$ mutations.

The $C_{154}T$ Mutation Is Not Restricted to PHI Patients

To verify that the loss of the Sty I site in all eight patients with mAGT is due to the same mutation, the first exon PCR product from patients A–L was screened by hybridization to allele-specific oligonucleotides (ASOs) corresponding to the normal and patient A AGT cDNA sequences around nucleotide 154. The results, shown in Fig. 5 *b*, confirm that all the patients with mAGT do carry the $C_{154}T$ mutation, whereas patients I–L do not. Our use of this technique was extended to screen first exon PCR product from a range of controls, and a sample of the results is also shown in Fig. 5 *b*. This revealed that the $C_{154}T$ mutation is present at an allelic frequency of 5–10% in the normal population. Of 60 individuals screened, 52 were homozygous for the normal allele, 7 were heterozygous for the $C_{154}T$ mutation, and 1 was homozygous for the mutation. While this clearly implies that the $C_{154}T$ mutation is insufficient to cause PHI, it does not necessarily indicate that this mutation is not causally related to AGT rerouting, since it has not been established that rerouting per se is sufficient to cause disease, and the subcellular distribution of AGT in the controls is unknown.

The $G_{630}A$ Mutation Is Restricted to PHI Patients with mAGT

The high allelic frequency of the $C_{154}T$ mutation in the normal population suggested that the other mutations observed in the cDNA of patient A may be necessary for the onset of disease in patients with mAGT. These mutations, $G_{630}A$ and $A_{1142}G$, cause a glycine to arginine conversion at residue 170 and an isoleucine to methionine conversion at residue 340, respectively. The distribution of these mutations among the PHI patients and a subset of the controls previously characterized for the $C_{154}T$ mutation was established by screening appropriate PCR products with allele-specific oligonucleotide probes corresponding to the respective normal and mutant sequences (Fig. 5, *c* and *d*). This revealed that for all the PHI patients in this study (A–K) the patterns of distribution of the $G_{630}A$ and $A_{1142}G$ mutations are identical to that of the $C_{154}T$ mutation. Patients G and H are homozygous for all three mutations, whereas patients A–F are heterozygous for each mutation, and patients I–K (as well as L) are homozygous for the normal sequence at all three sites. The linkage between the $C_{154}T$ and $A_{1142}G$ mutations extends to the normal individuals included in this study. In 41 of 42 such control subjects (including one homozygous and seven heterozygous for the $C_{154}T$ mutation) the pattern of occurrence of the $A_{1142}G$ mutation matched that of the $C_{154}T$ mutation. The exception was an individual (N_{24}) homozygous for the normal sequence at position 154, but heterozygous for the $A_{1142}G$ mutation. In contrast, the $G_{630}A$ mutation is completely absent from these 42 normal individuals (Fig. 5 *c*). The implication of this is that whereas the combined effects of the $C_{154}T$ and $A_{1142}G$ mutations are insufficient to cause PHI, even when present on both alleles, the additional presence of the $G_{630}A$ mutation is of significant importance in the determination of disease state in PHI patients A–H. This suggests a role for the $G_{630}A$ mutation in AGT rerouting. However, since the subcellular distribution of AGT in the normal controls is unknown due to the nonavailability of liver biopsies, and since it has not been established whether AGT rerouting per se is sufficient to cause disease, this data does not rule out the possibility that the role of the $G_{630}A$ mutation in the onset of disease is not directly related to AGT rerouting.

The $G_{630}A$ Mutation Is Important for AGT Rerouting

Towards the completion of this study a patient with primary hyperoxaluria of unknown etiology (possibly oxalate hyperabsorption) was brought to our attention. In this individual PHI was excluded on the basis of normal hepatic AGT activity (Table I, patient *M*). ASO screening of the AGT gene in this patient revealed that she was homozygous for the mutant allele carrying the $C_{154}T$ and $A_{1142}G$ mutations, but lacked the $G_{630}A$ mutation (Fig. 6 *b*). To investigate the subcellular distribution of AGT in this individual immunoelectron mi-

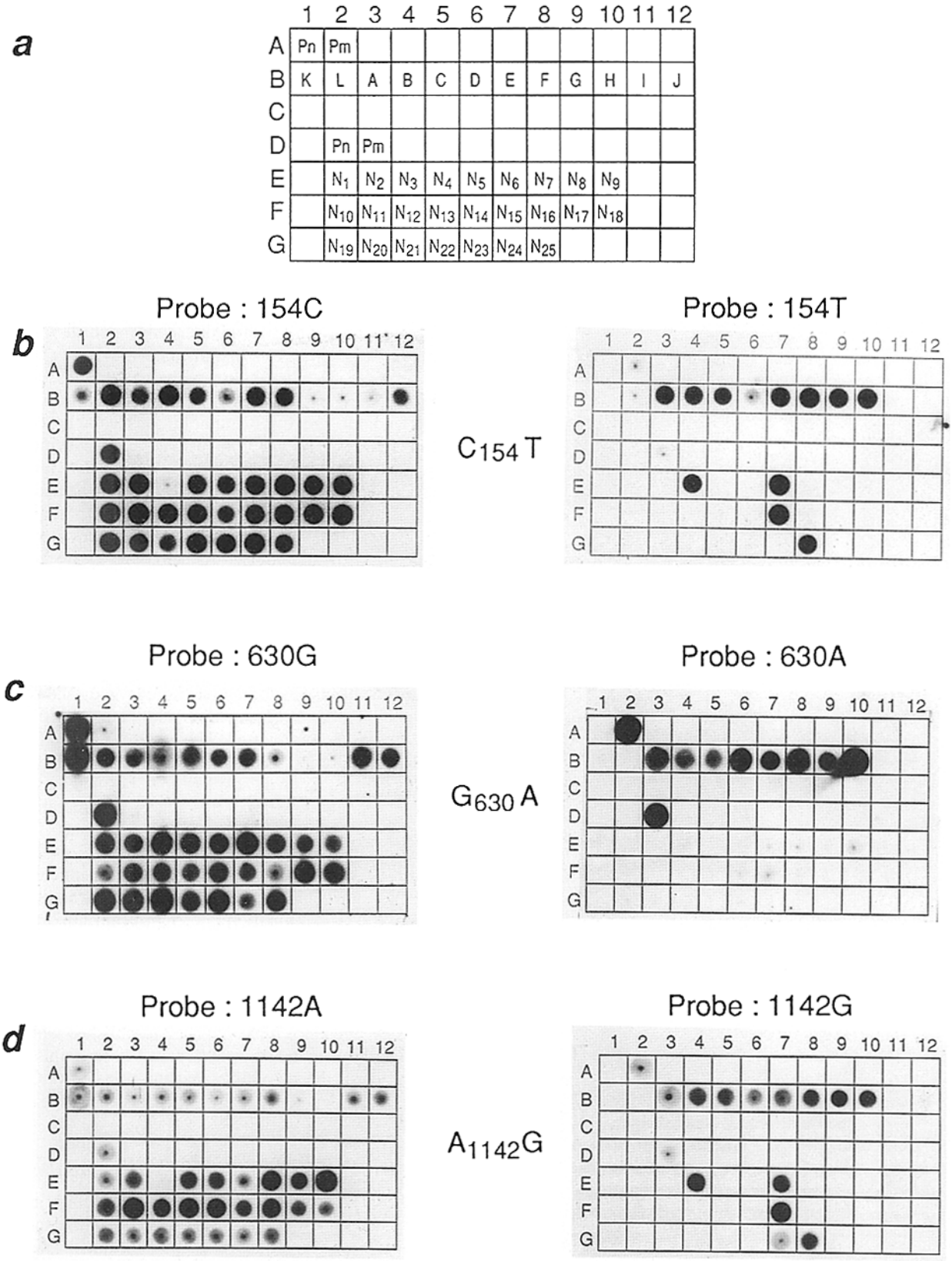

Figure 5. Dot blots of PCR product probed with ASO. (*a*) Grid showing the source of the PCR product corresponding to each position on the dot blots. P_n, cloned AGT cDNA (normal). P_m, cloned AGT cDNA (patient *A*). *A–L* are the patients in Table I. N_1–N_{25} are controls. (*b*) The $C_{154}T$ mutation. The probes cover nucleotides 147–161 of the cDNA sequence. (*c*) The $G_{630}A$ mutation (nucleotides 623–637). (*d*) The $A_{1142}G$ mutation (nucleotides 1,135–1,149). The sequences of the probes were as follows. *154C* = 5′-ACCCCCCCC-AAGGCC-3′; *154T* = 5′-ACCCCCCTCAAGGCC-3′; *630G* = 5′-TGGCTTCGGGGAACT-3′; *630A* = 5′-TGGCTTCAGGGAACT-3′; *1142A* = 5′-ACGTCATAGACCACT-3′; *1142G* = 5′-ACGTCATGGACCACT-3′; In each case the oligonucleotide probes are 15mers, differing only at the central residue. The generation of the PCR product for the blots in *b*, *c*, and *d* is described in the text.

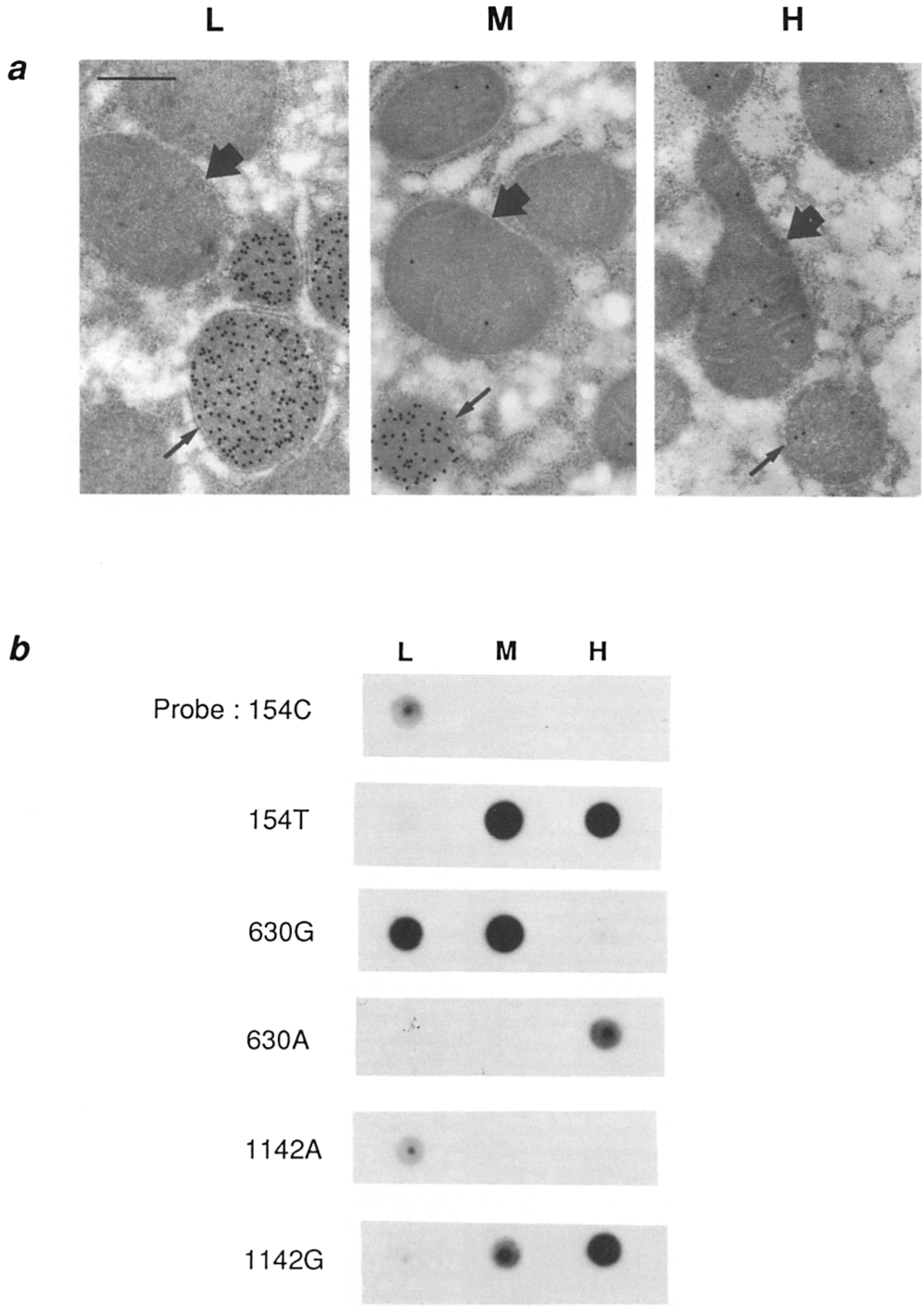

Figure 6. Comparison of the patterns of subcellular AGT distribution in three patients with different AGT alleles. (*a*) Protein-A gold Immunoelectron microscopy. ⟶, peroxisomes; ➡, mitochondria. *L*, *M*, and *H*, patients (see Table I for the patient details and Danpure et al. [1989] for the method) (*b*) ASO hybridizations. The PCR products and probes are described in the text and the legend to Fig. 5. Bar (*a*), 0.5 μm.

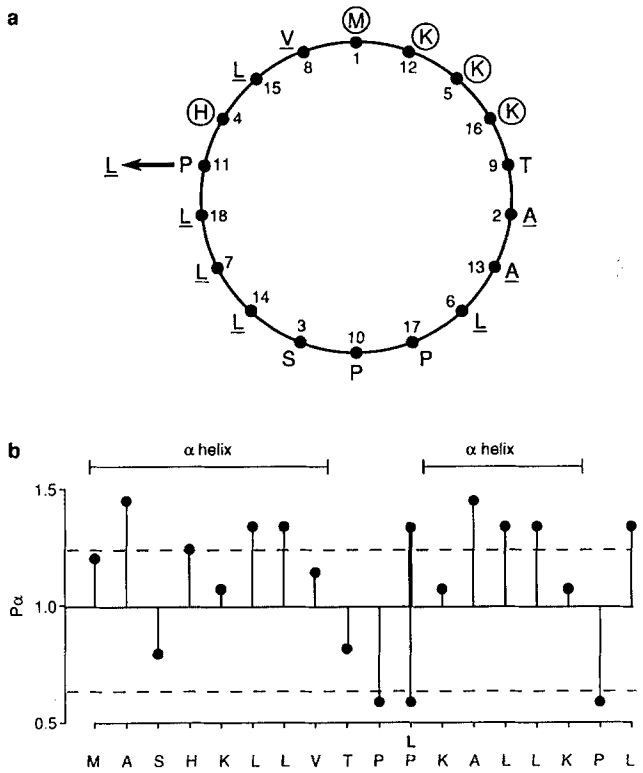

Figure 7. Predicted structural properties of the amino-terminal region of normal and mutant AGT. (*a*) Helical wheel analysis. Residues 1–18 of normal AGT are plotted as described by Schiffer and Edmundson (1967). Positively charged residues are circled and hydrophobic residues underlined. The arrow shows the proline to leucine substitution caused by the $C_{154}T$ mutation. (*b*) Predicted secondary structure. The alpha helix forming potential of residues 1–18 of normal AGT are plotted according to Chou and Fasman (1974). The bold bar at residue 11 shows the alteration in predicted structure caused by mutation $C_{154}T$.

croscopy was performed on a liver biopsy (Fig. 6 *a*). This revealed that although the majority (>90%) of immunoreactive AGT protein was peroxisomal, a small (<10%) but significant proportion of the protein was located in the mitochondrion. This contrasts with the situations in controls, where there is no detectable mitochondrial labeling and PH1 variants with mAGT, where >90% of immunoreactive AGT is mitochondrial (Fig. 6 *a*). A possible explanation of this pattern of distribution is that the appearance of mAGT and loss of pAGT in patients A–H are caused by two distinct but related events. Generation of the MTS, which is dependent upon the $C_{154}T$ and/or $A_{1142}G$ mutations, but not the $G_{630}A$ mutation, will lead to diversion of a small proportion of AGT protein to the mitochondrion, presumably through generation of a weak MTS. However, the $G_{630}A$ mutation, possibly through interference with the peroxisomal targeting and/or retention of AGT, is also required to achieve the pattern of AGT distribution observed in patients A–H (i.e., 90% of AGT within the mitochondria).

Discussion

Mutations in the AGT Gene of Patients with mAGT

The results presented in this paper show that the targeting of AGT to the mitochondrion instead of the peroxisome, which is, at least in part, responsible for disease in up to one-third of patients with PH1, is closely correlated with the presence and expression of an allele encoding AGT protein which differs from the normal protein at three positions, in each case by a single residue. The mutations $C_{154}T$ and $A_{1142}G$, which cause a proline to leucine substitution at residue 11 and an isoleucine to methionine substitution at residue 340, respectively, appear to cosegregate as an allele which is not only common to all PH1 patients with mAGT, but also present in the normal population at an allelic frequency of 5–10%. Such a high allelic frequency implies that as much as 1% of the population could be homozygous for these two mutations. Given that the incidence of PH1 is estimated to be very low, probably <0.002% of live births, this strongly suggests that expression of an allele(s) carrying these two mutations is insufficient to fully account for disease in PH1 patients with mAGT.

The third mutation, $G_{630}A$, which causes a glycine to arginine conversion at residue 170, appears to postdate the $C_{154}T$ and $A_{1142}G$ mutations, having originally arisen within an allele carrying these latter two mutations. The resultant allele, with the $G_{630}A$ mutation flanked by $C_{154}T$ and $A_{1142}G$, is common to all PH1 patients with mAGT, but absent from all the PH1 patients without mAGT and 42 control subjects (whose subcellular distribution of AGT is unknown, but is presumed to be mostly or entirely peroxisomal) included in this study. While this clearly implicates $G_{630}A$ as being important in the development of disease in PH1 patients with mAGT, it is not yet clear whether this mutation alone is sufficient to cause disease, or whether $C_{154}T$ and/or $A_{1142}G$ are also necessary.

Implications for AGT Targeting

Of the three mutations, $C_{154}T$ appears to be the most likely to be directly causally related to the generation of a MTS. The proline to leucine conversion caused by this mutation lies within the amino-terminal region of the protein where, by analogy with the vast majority of MTSs (Schatz, 1987), the MTS of the mutant AGT might be expected to reside. In addition, the amino acid sequence of AGT in this region displays the salient features shared by almost all amino-terminal MTSs, namely an absence of acidic amino acids but a high proportion of basic amino acids, distributed such that the structure of the signal peptide, which is thought to be alpha-helical in most cases, is markedly amphiphilic (Roise and Schatz, 1988; von Heijne, 1986; Lemire et al., 1989). When the first 18 amino acids of normal AGT are subjected to helical wheel analysis (Fig. 7 *a*) (Schiffer and Edmundson, 1967), the structure shows clear amphiphilicity. That this sequence does not act as a MTS is likely to be due largely to the proline–proline doublet at residues 10–11. Whereas the sequences either side of these prolines would be predicted to favor an alpha-helical conformation (Fig. 7 *b*) (Chou and Fasman, 1974), the helix destabilizing effect of two adjacent prolines virtually abolishes any possibility of this region of AGT existing as a continuous alpha-helical domain. Indeed, of 37 pro–pro doublets in proteins of determined structure, none exist in the central portion of an alpha-helix (MacArthur, M. W., and J. M. Thornton, personal communication). The predicted structural properties of this region of the mutant protein, with proline replaced by leucine at residue 11, are

very different. The sequence still plots as an amphiphilic alpha-helix, but in this case it seems much more likely that such a structure might exist. Single proline residues do occasionally exist within alpha helices when they are flanked by sequences that favor such a conformation (Barlow and Thornton, 1988). The only significant effect of single prolines in helices is the introduction of a kink of ~26 degrees (Barlow and Thornton, 1988), which would be insufficient to disrupt the amphiphilicity of the putative alpha helix at the amino terminus of mutant AGT. This is consistent with the results of a recent investigation by Lemire et al. (1989) in which the predicted helical amphiphilicity of a range of peptides encoded by random sequence oligonucleotides was compared to the functional mitochondrial targeting ability of these peptides. This comparison revealed an overall correlation between predicted helical amphiphilicity and functional mitochondrial targeting ability. However, peptides predicted to have significant helical amphiphilicity which also contained multiple helix-breaking residues were generally totally inactive as MTSs. This is highlighted by the observation that of the 80 peptides analyzed by these workers, none of the 60 sequences with mitochondrial targeting ability included Pro–Pro doublets, whereas 2 of the 20 nonfunctional peptides did. In contrast, 16 of the 60 functional peptides included a single proline, and 1 included 2 nonadjacent prolines, suggesting that single prolines can be accommodated within amphiphilic alpha-helical MTSs.

Although the mutation at residue 11 can be envisaged as being causally related to the generation of an amino-terminal MTS in AGT, it is not clear whether such an event would be sufficient not only to result in targeting to the mitochondrion, but also to abolish targeting to the peroxisome. The levels of residual peroxisomal AGT in PH1 patients with rerouted enzyme is very low (0.3–3% normal) (Danpure et al., 1989; unpublished observations), implying that the ability to accumulate AGT in peroxisomes is severely impaired in these individuals. Whether this loss of peroxisomal targeting activity is an unavoidable consequence of acquisition of a functional MTS, or alternatively whether it is a prerequisite for the accumulation of significant levels of AGT in the mitochondrion is not known.

There are two possible ways in which generation of an amino-terminal MTS could affect peroxisomal targeting. One possibility is that the same mutation which generates the MTS also destroys the peroxisomal targeting sequence (PTS). Although the molecular basis of targeting of AGT to the peroxisome is not known, this seems unlikely in view of the increasing evidence in favor of carboxy-terminal PTSs (Gould et al., 1987, 1988, 1989, 1990; Miyazawa et al., 1989). The extreme carboxy-terminal sequence of AGT, as deduced from the cDNA sequence (-KKKL) shows similarities to the carboxy-terminal sequence of firefly luciferase (-KSKL), the last three amino acids of which have been shown to be a necessary and sufficient PTS (Gould et al., 1989). Whether this sequence acts as a PTS for AGT is in some doubt due to the finding that mutation of the -KSKL sequence of firefly luciferase to -KKKL appears to abolish peroxisomal targeting ability in transfected monkey kidney cell cultures (Gould et al., 1989). Nevertheless, the likelihood of the amino-terminal sequence of AGT being directly involved in peroxisomal targeting remains small. No amino-terminal PTSs have been reported to date, although it has been suggested (Arakawa et al., 1987) that an amino-terminal region of rat peroxisomal 3-oxoacyl CoA thiolase which is absent from the mitochondrial isozyme may play an indirect role in peroxisomal targeting by masking the adjacent region of the protein, which is the putative MTS of the mitochondrial isozyme.

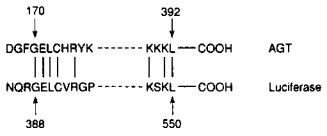

Figure 8. Putative peroxisomal targeting sequences of AGT. Comparison with firefly luciferase. The sequence around the glycine residue at position 170 (which is replaced by an arginine by the $G_{630}A$ mutation) shows some homology to an internal sequence of firefly peroxisomal luciferase. The COOH-terminal sequences of these proteins are also similar, although the lysine residue third from the end appears to prevent this sequence acting as a peroxisomal targeting sequence (Gould et al., 1987).

A second possible mechanism by which acquisition of an amino-terminal MTS could disrupt peroxisomal targeting is via a hierarchical system of expression of the two targeting sequences, whereby the amino-terminal MTS is recruited by mitochondria before synthesis and folding of the internal or carboxy-terminal PTS. This would require rapid and efficient commitment to the mitochondrial import pathway to achieve the low level of pAGT seen in patients with mAGT, which appears to be at odds with the rather low steady-state levels of mAGT in these patients. For instance, patients A–C, each of whom express a single allele carrying the $C_{154}T$ mutation and appear to have ~50% normal levels of AGT mRNA (Takada et al., 1990; Purdue, P. E., unpublished observations), have only 5.6–8.7% normal enzyme levels and much reduced levels of immunoreactive protein. There is no reason to expect that the protein is unstable in the mitochondrion, since it appears to retain its enzymatic activity (Danpure et al., 1989).

It is perhaps more reasonable to expect that the adventitious MTS formed by the proline to leucine conversion at residue 11 is a rather inefficient targeting sequence which, when present together with an intact functional PTS, will only reroute a small proportion of the AGT to the mitochondrion. In this case, the loss of AGT peroxisomal targeting and/or retention activity might be expected to lead to an increase in the functional expression of the MTS. Whether this is the situation for the mutant AGT in patients with mAGT remains to be resolved, but there is some preliminary evidence that the $G_{630}A$ mutation is also directly involved in the rerouting of AGT. This is based upon the observation that there is a dual localization of AGT in the liver of an individual who is homozygous for the $C_{154}T$ and $A_{1142}G$ mutations, but lacking the $G_{630}A$ mutation. The proportion of AGT rerouted to the mitochondrion in this individual is small (<10%), but significant, and this indicates that at least two mutations are required to give rise to the pattern of AGT sorting found in PH1 patients with mAGT. This suggests that the $C_{154}T$ (and/or $A_{1142}G$) mutation(s) is sufficient for the generation of an inefficient MTS, but that the additional presence of the $G_{630}A$ mutation is required to effect functional expression of this targeting sequence to the near exclusion of peroxisomal import.

The most likely hypothesis to explain this is that the $G_{630}A$ mutation affects peroxisomal targeting and/or retention of AGT. Small et al. (1988) have reported that internal sequences are involved in the peroxisomal targeting of Acyl-

CoA oxidase in the yeast *Candida tropicalis*, and although this is the only report to date of an internal PTS, a number of glycosomal enzymes of the *Trypanosomatidae* are apparently targeted by internal highly charged sequences which are expressed as topogenic signals on the surface of the molecule (Opperdoes, 1988). It has been suggested that a similar mechanism could be involved in targeting of peroxisomal proteins (Opperdoes, 1988), a hypothesis that is not incompatible with the identification of COOH-terminal PTSs in firefly luciferase and several other peroxisomal proteins (Gould et al., 1987, 1988, 1989; Miyazawa et al., 1989), since a number of peroxisomal proteins (possibly including human AGT) do not have an SKL or equivalent variant near the COOH-terminus (Borst, 1986, 1989).

The glycine to arginine change induced by the $G_{630}A$ mutation lies within a stretch of 44 amino acids (residues 133–176; Fig. 1 *c*) that are identical to the corresponding region of rat AGT, whose dual localization in peroxisomes and mitochondria is consistent with the presence of an internal or carboxy-terminal PTS (Takada et al., 1990; Oda et al., 1987). This degree of conservation suggests that this region of the protein is crucial to the functional expression of pAGT and that the maintenance of activity and/or import would be fairly intolerant to mutations within this region. Although the AGT activities in the mitochondrial variant PH1 patients are fairly low (<30% normal) they are all roughly proportional to the level of immunoreactive protein, which indicates that the pathological effect of the $G_{630}A$ mutation is probably not a result of impaired enzymatic activity of the residual mitochondrial protein.

It is our opinion that these observations suggest a role for the sequence around residue 170 in the peroxisomal targeting of AGT. It is interesting to note that this sequence, which is reasonably highly charged, shows partial homology to an internal sequence of firefly luciferase (Fig. 8) (de Wet et al., 1987). The identification of this sequence, and that around residue 11, as candidate peroxisomal and mitochondrial targeting sequences, respectively, will be of great help in the design of in vitro mutagenesis experiments to define these sorting signals in detail.

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