Molecular genetics of disorders of haem biosynthesis

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Introduction
Haem is synthesised in all animal cells from succinyl CoA and glycine by a sequence of reactions catalysed by eight enzymes.1 Absence of any one of these enzymes is incompatible with life because haem is an essential component of respiratory proteins. Partial deficiencies occur, however, and cause disease (table 1).1,2 The activity of 5-amino- laevulinate (ALA) synthase, the first and rate controlling enzyme of the pathway, is decreased in the bone marrow in hereditary sideroblastic anaemias,3 while deficiency of each of the subsequent enzymes produces a particular type of porphyria (table 1).1,2 The enzyme defect in each porphyria is inherited (table 1), except in type I porphyria cutanea tarda which appears to be an acquired disorder. The clinical features and laboratory diagnosis of the porphyrias have been reviewed.1,2

Genes or cDNAs have now been isolated and characterised for all but two of the human enzymes (table 2). These advances have been followed rapidly by identification of mutations in the corresponding diseases and by the application of DNA techniques to the management of families with these conditions. This review summarises current information about the molecular genetics of each disorder.

Hereditary sideroblastic anaemias
The hereditary sideroblastic anaemias are a mixed group of disorders in which the most frequent pattern of inheritance indicates linkage to the X chromosome. Bone marrow ALA synthase activity is usually decreased.3 Pyridoxal phosphate is an essential cofactor for this enzyme and patients may respond to treatment with pyridoxine.3

Mammals have two ALA synthase genes (table 2). The X chromosome gene encodes ALAS2 which is expressed only in erythroid cells while that on chromosome 3 encodes a housekeeping isoenzyme (ALAS1) that is found in all tissues.4,5 The C-terminal portion of ALAS2 (exons 5–11) has 73% sequence identity with ALAS1 and contains the catalytic domain. Exons 2–4 of the ALAS2 gene encode the N-terminal signal sequence that directs mitochondrial import and an intervening section that undergoes differential pre-mRNA splicing to generate two isoforms (table 2).4 The functional importance of this pattern of processing has not been established.4 Expression of the ALAS2 gene is regulated at transcriptional and translational levels. Its promoter region, in common with those of the erythroid PBGD,20 ferrochelatase,20 and β globin genes,21 contains sequences that interact with the trans-acting erythroid specific factors, GATA-1 and NF-E2, and regulate transcription during erythroid differentiation.3 The 5' untranslated region (UTR) of ALAS2 mRNA, unlike ALAS1 mRNA, contains a sequence structure motif that closely resembles the iron responsive elements (IREs) found in the 5' UTR of ferritin mRNA and the 3' UTR of transferrin receptor mRNA.3,22 Iron deficiency leads to high affinity binding of an IRE-binding protein to these elements with consequent inhibition of translation of ALAS2 and ferritin mRNAs and stabilisation of transferrin receptor mRNA.22,23 A mechanism that allows co-ordination of protoporphyrin formation with iron supply. The presequences of both ALAS isoenzymes also contain a conserved, haem regulatory motif which is involved in inhibition by haem of their transport into mitochondria.24

The discovery that ALAS2 maps to the X chromosome prompted a search for mutations in this gene in sideroblastic anaemia. A T→A transition that converts isoleucine to asparagine has recently been identified in a man with severe, pyridoxine responsive sideroblastic anaemia.25 This mutation is in a highly conserved region of exon 9 that is close to the putative pyridoxal phosphate binding lysine residue and thus may impair binding of the cofactor. This man had no family history of anaemia but a second mutation in the ALAS2 gene has now been found in a family with X linked pyridoxine responsive sideroblastic anaemia.25 Because the low level of expression of ALAS1 in erythroid cells is unlikely to be sufficient by itself for erythropoiesis,5 other inherited ALAS2 defects in sideroblastic anaemia are similarly likely to result from point mutations that modify but do not abolish enzyme activity. Not all families with X linked sideroblastic anaemia have defects in the ALAS2 gene. Combined use of a highly polymorphic dinucleotide repeat in intron 7 of the gene and other X chromosome polymorphic markers in linkage studies has shown that there are at least two X loci for sideroblastic anaemia.26 Overall, these studies suggest that analysis of the ALAS2 gene is likely to improve both diagnosis and classification of these anaemias and may even reveal a role for somatic mutation of this gene in some acquired forms.

Autosomal dominant porphyrias
The enzyme defects in most porphyrias are inherited in an autosomal dominant manner (table 1). The clinical penetrance of these
disorders, however, is low; in each condition more than 80% of those who inherit the genetic defect never have symptoms and most of these have no detectable biochemical abnormality apart from enzyme deficiency. The frequency of the genes for these conditions in the population is sufficiently high for homozygous forms of each disorder to occur without consanguinity and for occasional co-inheritance of two separate porphyrias.

**ACUTE INTERMITTENT PORPHYRIA**

Acute intermittent porphyria (AIP) is the commonest of the acute hepatic porphyrias and at present is the only one that has been investigated at the DNA level. It is characterised by life-threatening acute neurovisceral attacks that are frequently precipitated by drugs, calorie restriction, or alcohol. Detection of asymptomatic gene carriers so that they can be advised to avoid known precipitants of acute attacks is an important part of the management of AIP families.

AIP is caused by porphobilinogen deaminase (PBGD) deficiency. Enzyme activity is close to 50% of normal, reflecting expression of the normal gene allele to the mutant gene. As with ALA synthase, there are separate erythroid and housekeeping isoenzymes of PBGD deaminase (table 2); current evidence suggests that there are no tissue specific isoenzymes for the other enzymes of the pathway. In contrast to ALA synthase, however, PBGD is encoded by a single gene (table 2) which is transcribed from separate erythroid specific and housekeeping promoters. The erythroid promoter lies in intron 1 and transcription is initiated 5' to exon 2. Translation of erythroid mRNA starts in exon 3 so that the erythroid isoenzyme contains sequence encoded by exons 4–15 and a 3' section of exon 3. RNA for the housekeeping isoenzyme is transcribed from a promoter 5' to exon 1 and then spliced to exclude exon 2. Translation then proceeds from a start codon in exon 1 so that the housekeeping isoenzyme is 17 amino acids longer at the N-terminus than the erythroid form. Mutations that affect both isoenzymes would thus be predicted to occur in exons 3–15 or in intronic sequences that determine the structure or level of expression of the corresponding mRNA.

Three subtypes of AIP can be distinguished by measurement of erythrocyte PBGD: an uncommon (less than 5% of families) form in which erythrocyte PBGD is normal with only the ubiquitous isoenzyme being defective; a form in which the product of the mutant allele cross-reacts immunologically with antiserum to normal enzyme but has absent or substantially impaired catalytic activity (CRIM positive); and CRIM negative AIP in which immunoreactivity and catalytic activity are decreased in parallel. Mutations have been identified in all three subtypes. In the non-erythroid subtype two mutations that affect the splicing of exon 1 during pre-mRNA processing and thus impair the formation of mRNA for the ubiquitous isoenzyme only have been found in seven of 10 unrelated patients. About 15% of unrelated patients with AIP have the CRIM positive subtype. Studies from the Netherlands and France suggest that about 75% of these patients have mutations that lead to replacement of either of two conserved arginine residues (R167 and R173) in exon 10 by glutamine or tryptophan with one mutation (G→A at base position 500; R167Q) being more frequent than the others. These mutations severely impair but do not abolish enzyme activity. Interestingly, the only two unrelated cases of homozygous AIP that have been reported were both compound heterozygotes for three of the CRIM positive exon 10 mutations, suggesting that their frequency may be higher than the prevalence of overt CRIM positive AIP would indicate. The structure of PBGD deaminase is highly conserved with over 45% amino acid sequence identity between the *Escherichia coli* and human enzymes. X-ray analysis of the crystal structure of the *E coli* enzyme shows that the arginine residues corresponding to these two residues in exon 10 of human PBGD and a third one in exon 3, which is the site of another CRIM positive mutation (R26H) in AIP, form salt bridges with the acidic side chains of the dipyrro-

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Gene</th>
<th>cDNA in base pairs (length of ORF)</th>
<th>Expression</th>
<th>Chromosome</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>ALAS1</td>
<td>Ferrochelatase (PC)</td>
<td>1920(160)</td>
<td>U</td>
<td>3p21</td>
<td>45</td>
</tr>
<tr>
<td>ALAS2</td>
<td>23kb, 11 exons</td>
<td>1746(147)</td>
<td>E</td>
<td>Xp11-12</td>
<td>44</td>
</tr>
<tr>
<td>PBGS</td>
<td>990</td>
<td>1635(147)</td>
<td>E</td>
<td>Xp11-12</td>
<td>44</td>
</tr>
<tr>
<td>PBGD</td>
<td>10kb, 15 exons</td>
<td>1032</td>
<td>U</td>
<td>1q41-q24-2</td>
<td>8, 33</td>
</tr>
<tr>
<td>UROS</td>
<td>1083</td>
<td>1q41-q24-2-q26-3</td>
<td>U</td>
<td>1q41-q24-2-q26-3</td>
<td>12, 34</td>
</tr>
<tr>
<td>UROD</td>
<td>1101</td>
<td>U</td>
<td>1p34</td>
<td>15, 36</td>
<td></td>
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<tr>
<td>COPROX</td>
<td>9</td>
<td>U</td>
<td>14</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>PROTOX</td>
<td>45kb, 11 exons</td>
<td>1269(162)</td>
<td>U</td>
<td>18q21-3</td>
<td>19, 20</td>
</tr>
</tbody>
</table>

ORF: open reading frame. Number of bases encoding N-terminal signal peptides for mitochondrial import are shown in parentheses.

U: ubiquitous; E: erythroid cells only.
methane cofactor that lies within the cleft between domains 1 and 2. CRIM positive AIP thus seems to result from mutation of residues that are both important for catalysis and located deep within the active site cleft so that their substitution does not alter surface epitopes.

The commonest subtype, CRIM negative AIP, seems to be more heterogeneous than the others. At least 20 base substitutions, insertions, or deletions that produce missense, nonsense, or splice defective mutations have been identified. Most have been found in only one or two families but a non-sense mutation in exon 10 (Q198 stop) is common in Sweden, where it seems to have spread through a founder effect from a family originating in Lapland and to explain the high frequency of overt AIP in this country (1 in 1500 in the north). About one third of Dutch families with AIP share an R116Q mutation, again suggesting a founder effect.

DNA analysis has an important practical application in AIP for the identification of asymptomatic gene carriers. Several studies have shown its superiority for this purpose over conventional biochemical techniques, which have a number of drawbacks. At present, the following would seem a reasonable strategy for detecting carriers by DNA techniques. For families in which the mutation is known, methods based on polymerase chain reaction (PCR) for their detection will give unequivocal assignments. Similarly, patients with the non-erythroid or CRIM positive subtypes, in which certain mutations are common, or from areas where one mutation is frequent, can be rapidly screened for the presence of these mutations. For those families in which the mutation is unknown, which at present means most, two approaches are possible: gene tracking using linkage to intragenic polymorphisms or identification of the mutation in the proband followed by direct screening of relatives. Seven intragenic two allele polymorphisms have been identified in the PBGD gene (table 3). Three are in complete linkage disequilibrium and there is partial linkage disequilibrium between others so that the full number of haplotypes is not observed. Most patients, however, are heterozygous at one allele or more and study of their families is therefore potentially informative. The main disadvantage is that it is often impossible to establish linkage because not enough unequivocally affected relatives are available for investigation. An alternative but more laborious approach is to identify the mutation in genomic DNA or cDNA from the family under investigation by using a rapid scanning method, such as denaturing gradient gel electrophoresis (DGGE), to locate the mutation, followed by direct sequencing.

Table 3 Human PBGD deaminase gene: intragenic two-allele polymorphisms

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Site</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/T</td>
<td>exon 1</td>
<td>0-63(C)</td>
</tr>
<tr>
<td>MspI</td>
<td>intron 1</td>
<td>0-40</td>
</tr>
<tr>
<td>PstI</td>
<td>intron 1</td>
<td>0-60</td>
</tr>
<tr>
<td>ApaLI, Snol</td>
<td>intron 1</td>
<td>0-40</td>
</tr>
<tr>
<td>BstNI, ScrFl</td>
<td>intron 1</td>
<td>0-55</td>
</tr>
<tr>
<td>GA</td>
<td>intron 3</td>
<td>0-60(G)</td>
</tr>
<tr>
<td>G/T</td>
<td>exon 10</td>
<td>0-65(G)</td>
</tr>
</tbody>
</table>

For RFLPs, the frequency is that of the uncut allele. MspI, PstI, and ApaLI RFLPs are in complete linkage disequilibrium.

OTHER AUTOSOMAL DOMINANT PORPHYRIAS
Porphyria cutanea tarda is a cutaneous porphyria that results from decreased activity of uroporphyrinogen decarboxylase (UROD) in the liver. Its pathogenesis is complex but one form (type II), which accounts for about 20% of cases, is associated with autosomal dominant inheritance of UROD deficiency in all tissues. More severe UROD deficiency (activity less than 25% of normal) characterises the much rarer, presumed homozygous form of type II porphyric cutanea tarda, hepatoerythropoietic porphyria (HEP), which is biochemically heterogeneous. Five point mutations and a deletion of the UROD gene have now been identified in patients with inherited deficiencies of this enzyme. Contrary to the anticipated relationship, mutations identified in patients with HEP have not yet been found in those presenting with type II. For example, the G281E mutation, which decreases enzyme stability and was present in one Tunisian and five Spanish families with HEP, was not detected in 12 type II porphyria cutanea tarda families from Spain (Roberts AG, Elder GH, unpublished observations). The UROD defect in type II porphyria cutanea tarda is CRIM negative and it may be that most of the causative mutations are too severe for homozygotes to survive. Garey et al found that five of 22 North American families with type II porphyria cutanea tarda had a splice site mutation producing deletion of exon 6 from mRNA and encoding an inactive, unstable truncated protein. This mutation was not present in 25 European families (Roberts AG, Elder GH, unpublished observations), which together with other studies of the frequency of mutant uroporphyrinogen decarboxylases suggests extensive molecular heterogeneity in this group of disorders.

Progress has also been made in understanding the molecular basis of erythropoietic protoporphyria (EPP). This disorder is characterised by severe photosensitivity; in a few patients accumulation of protoporphyrin in hepatocytes leads to irreversible liver failure. In most families EPP is inherited as an autosomal dominant trait with low penetrance, but other modes of inheritance have been postulated. Animal models of the disease, whether occurring naturally in cattle, or...
produced by ethynitrosourea mutagenesis in mice,\textsuperscript{49} are inherited as autosomal recessive traits. Accurate identification of carriers is important if families are to be counselled adequately.

EPP results from deficiency of ferrochelatase (table 1). The activity of this enzyme is less than the half-normal that would be expected for an autosomal dominant disorder.\textsuperscript{12,27} Functional ferrochelatase may be a homodimer\textsuperscript{32} or if this is so, interaction between normal and mutant subunits might decrease activity by greater than 50%.\textsuperscript{55,58} Functional point mutations that cause EPP have recently been identified in the ferrochelatase gene. One patient with the rare homozygous form of EPP was a compound heterozygote for two different missense mutations.\textsuperscript{39} In three other families autosomal dominant inheritance of mutations causing aberrant splicing\textsuperscript{60-61} or an amino acid substitution leading to decreased activity\textsuperscript{84} were found. It thus seems likely that carrier detection in EPP will be complicated by extensive molecular heterogeneity. Linkage analysis in EPP families, however, should be facilitated by the recent discovery of a dinucleotide repeat polymorphism in intron 2 of the gene.\textsuperscript{58} At present, there is no evidence that particular genotypes are associated with the propensity to develop liver disease.

**Autosomal recessive porphyrias**

Two types of porphyria are inherited in an autosomal recessive pattern: PBGS (5-aminolaevulinate dehydratase) deficiency porphyria and congenital erythropoietic porphyria (CEP) (table 1).

Patients from the four unrelated families with PBGS deficiency porphyria that have been described show substantial phenotypic variation, ranging from failure to thrive in infancy through the onset of acute attacks of porphyria soon after puberty, to subacute polyneuropathy at the age of 63.\textsuperscript{65} Patients with the infantile and teenage onset forms have been shown to be compound heterozygotes for four different missense mutations in the PBGS gene\textsuperscript{111,114}; expression studies show that three of these substantially decrease enzyme activity, but the other, which was present in a teenage patient,\textsuperscript{84} was less severe, suggesting some correlation between genotype and phenotype in this very rare condition.

Congenital erythropoietic porphyria is the least common of the main types of porphyria apart from PBGS deficiency. Typically, it presents in early infancy with severe skin lesions that progress to photomutilation which are accompanied by splenomegaly and haemolytic anaemia of variable severity. There is also a less severe form with onset after childhood.\textsuperscript{127} There are no reports of both forms occurring in the same family which suggests that they may be produced by different genotypes. Congenital erythropoietic porphyria is caused by decreased activity of UROS (table 1). Eight mutations have been identified in the UROS gene or cDNA in patients with congenital erythropoietic porphyria; one of these (C73R) accounts for about 20% of congenital erythropoietic porphyria alleles.\textsuperscript{66-67} Most patients are compound heterozygotes and no clear relationship between genotypes and the two different phenotypes has yet been established. Homozygotes for the C73R mutation, however, are severely affected while patients with a T228M mutation of one allele seem to have less severe disease.\textsuperscript{67} The infantile form of congenital erythropoietic porphyria is probably the only type of porphyria for which prenatal diagnosis should be available. DNA analysis should allow this to be achieved with greater precision than is currently possible.

**The future**

Several important problems in the pathogenesis and management of inherited disorders of haem biosynthesis now seem likely to be solved by the application of recombinant DNA methods. In the acute hepatic porphyrias, the way towards much more accurate detection of gene carriers is already clear from studies on AIP; similar progress for variegate porphyria and hereditary coproporphyria should follow once the relevant genes have been cloned. The influence of genetic factors in determining phenotypic variation and differences between families in the penetrance of disease is now likely to be explored. For example, genes that directly or indirectly influence the inducibility of ALAS1 in the liver may be important determinants of susceptibility to acute porphyria. Finally, transgenic animal experiments may help to solve outstanding problems, such as the mechanism of the acute porphyric attack, and lead to new treatments.


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