To our dear Paul

Always fondly remembered
with love in our hearts
and sadly missed
Human flavin-containing monooxygenase genes: characterization and chromosomal localization

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Abstract

The two human genes FMO2 and FMO5 are shown to be located on the long arm of chromosome 1 (1q) through the screening of human-rodent somatic cell hybrids by PCR.

Fifteen YAC clones shown to contain one or more FMO genes within their inserts were isolated from an ICRF YAC human genomic DNA library. Two clones were found to contain FMO1, FMO2, FMO3 and FMO4 within their inserts. All clones containing one or more of FMO1, FMO2, FMO3 or FMO4 were found by fluorescence in-situ hybridization (FISH) to human metaphase chromosomes to hybridize at 1q23-24. Four clones were found to contain FMO5: in each case, none of the other known FMO genes were also present. FISH to human metaphase chromosomes with these four clones revealed hybridization at 1q21.

Subjecting three of the YAC clones, two containing FMO5 and the other FMO1, FMO2, FMO3 and FMO4, to pulsed field gel electrophoresis, allowed estimation of their insert sizes.

Screening of an ICRF chromosome 1-specific cosmid library resulted in the isolation of five cosmid clones containing inserts with apparent homology to FMO sequences. Southern hybridization and FISH analysis, however, indicated that the clone inserts did not contain FMO genes. Rather, four inserts appeared to originate from heterochromatic regions and the other from 15q11-13.
Acknowledgments

Having completed my thesis, I can honestly say that this section is probably the hardest of all to write. PhD thesis acknowledgments have a habit of appearing sugary and over-emotional to the reader, due no doubt to the state of mind of the author in question. The relief that it really is all finally done, after all those years of sacrifice, hard slog in the laboratory and months of dawn-till-dusk grafting on the computer, can be emotionally overwhelming. I think only a fellow former PhD student can really appreciate exactly what an undertaking a PhD can be. How many ventures involve working at 9pm on Christmas eve in a lab so cold you can no longer feel your toes and then leave you financially impoverished into the bargain?

It is an impossible task to adequately thank every person who has contributed to this thesis over the years, so I apologize now to those whom I do not mention below. It is a fact that the work presented in the following pages could not have been achieved without the help, love and support of loved ones and friends. In that sense, this thesis is the joint effort of many and much of the credit is due others apart from myself.

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Chapter 1

An introduction to the enzymology and molecular biology of the flavin-containing monooxygenases and the scope of this thesis.
1.1 Introduction

Flavoproteins that catalyze the biotransformation of xenobiotics comprise a heterogeneous group of enzymes, including reductases, oxidases and monoxygenases. All possess a flavin prosthetic group, with its inherent isoalloxazine ring, in the form of either flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN), and this allows participation by these enzymes in a range of redox reactions.

The flavin-containing monoxygenases (FMOs) are a subset of this enzyme group. FMOs appear ubiquitous, having been found in virtually every nucleated mammalian cell examined in association with the endoplasmic reticulum (i.e. can be isolated from the microsomal fraction) (1, 2). There is even a prokaryotic equivalent of FMO, cyclohexane monoxygenase from *Acinetobacter*. However, despite cyclohexane monoxygenase being mechanistically similar to mammalian monoxygenases (3), it has an amino acid sequence only 25% identical to that of the mammalian FMOs (4), and so is generally not regarded as an FMO in the strictest sense. A flavoprotein with similarities to FMO has also been found within *Trypanosomi cruzi* (5). In mammals, FMO activity may be highest in the liver but is present in significant amounts in several extrahepatic organs such as lung, adrenal, kidney, thymus and brain (6-9). FMO activity was first described by Pettit et al. in 1964 (10) and we now know that the FMO enzymes are monomeric and range in molecular mass from 59000 to 63000. FMOs catalyze the NADPH-dependent monooxygenation (transfer of a single oxygen atom derived from molecular oxygen) of so-called soft nucleophilic atoms with an electron-rich centre, these being principally nitrogen, sulphur, selenium and phosphorus-containing functional groups. Prior to the discovery of the FMOs, it was assumed that most, if not all, NADPH-dependent xenobiotic heteroatom-containing compound oxidation was mediated by the microsomal cytochrome P-450 family of monoxygenases. Indeed, most of the studies to date on chemical and drug oxidation in humans focus on the role of the cytochromes P450 (11). With the passage of time, however, awareness has increased regarding the key role played by the FMOs in the conversion of nucleophilic amines, sulphides and other heteroatom-containing drugs and chemicals into relatively polar, readily excretable metabolites. A hepatic flavoprotein was first isolated and purified from hog liver in 1972 by Ziegler and coworkers (12), who had earlier characterized the same enzyme and termed it ‘N-oxidase’, or the ‘mixed function amine oxidase’, or ‘Ziegler’s enzyme’ after Professor Daniel Ziegler (13, 14). It soon became apparent that the term ‘amine oxidase’ was too
restrictive as FMOs, as they were to become known, were found to oxygenate substrates as diverse as hydrazines (15), phosphines (16), iodide (17), boron-containing compounds (17), sulphides (18, 19), selenides (20), as well as many tertiary and secondary amines (6, 18, 21) Thus, numerous pesticides, drugs (e.g. chlorpromazine, benzphetamine, nortriptyline, propylthiouracil, ethylmorphine), pollutants and compounds present within plant materials are potential FMO substrates (6, 18, 22-25). The realization that the FMOs were major participants in the metabolism of xenobiotics carried with it all of the inherent potential pharmacological and toxicological implications long associated with the P450s. Endogenous substrates and potential physiological roles for the FMO, however, appear so far to be limited in number, unlike the P450s. Duescher et al. (26) have shown that methionine is a substrate for three rabbit FMO enzyme forms (FMO3, FMO2 and FMO1, in order of increasing $K_m$), but not for another FMO enzyme form in the rabbit, FMO5: the resulting products being diastereometric sulphoxides. Although methionine sulphoxidation has been demonstrated in rat liver and kidney microsomes and appears exclusively mediated by FMOs (27), it is believed that the reaction is of biological significance only when toxic levels of methionine are present within the diet or in individuals with a genetic defect in the methionine transshuration pathway. Another endogenous substrate appears to be cysteamine. Cysteamine is efficiently converted to the disulphide, cystamine, which has itself been proposed to serve as an endogenous source of disulphide equivalents in the strongly reducing environment of the cell (24, 28, 29). It has been suggested that endogenous disulphides formed by FMO, such as cystamine, act as low molecular weight disulphide exchange agents, which may be needed for the naturation of proteins requiring a disulphide bridge for proper structure and function (28); Ziegler, 1993 #213; Ziegler, 1990 #458; Ziegler, 1977 #701. Recently, $S$-benzyl-$L$-cysteine has been added to the list of potential endogenous substrates for FMO, with the FMO enzyme form FMO1 being the major contributor to its $S$-oxidation (30). It is also possible that endogenous cysteine- and homocysteine-$S$-conjugates may function as FMO substrates (30), as may ethionine and selenomethionine, the latter of which was shown to inhibit methionine sulphoxidation in rat liver and kidney microsomes (31).

The universal prevalence of FMOs signifies a crucial metabolic role maintained by natural selection. Given their apparently limited role in endogenous metabolism, it would seem that their function has predominantly concerned facilitating the removal of unwanted chemicals internalized from the environment. Through monooxygenating their substrates, FMOs, like other so-called ‘phase I’ enzymes, convert the substrate into a more hydrophilic
molecule. In this manner, the substrate is prevented from accumulating within the numerous lipid regions of the organism and can be readily excreted, chiefly in the urine. Thus, even if not toxic in low amounts, the substrate is prevented from reaching concentrations that might eventually be so. It has long been recognized that in the case of the P450s, an inadvertent ‘side-effect’ of this essential process of enhanced xenobiotic elimination is the conversion, in some cases, of substrates to products with enhanced toxicity. This includes the generation of products capable of the covalent modification of cellular macromolecules, including DNA. As a result, the P450s have been the subject of scrutiny for their role in the etiology of mutagenesis and carcinogenesis. Mankind has exacerbated this undesirable ‘trade-off’ through the comparatively recent introduction into the environment of various pollutants, pesticides, drugs etc. that were never present during the evolution of this enzyme system and which could therefore have played no part in the processes of natural selection from which they arose. The question follows immediately as to what role the FMOs may have in this regard. Although FMOs and cytochromes P450 show activity toward many of the same substrates, usually, but not always, it can be said that oxidative metabolism of heteroatom-containing compounds by cytochrome P450-dependent processes leads to products with increased potential for toxic, mutagenic or carcinogenic properties. Alternatively, the FMOs generally convert lipophilic heteroatom-containing compounds to polar, readily excretable oxygenated metabolites that possess decreased toxic potential (32), although there are exceptions whereby FMOs contribute to the bioactivation of certain xenobiotic compounds to more reactive intermediates capable of eliciting a pathological response (33-37). Examples in this regard include 1,1-disubstituted hydrazines, N-alkylarylamines, alkylthiols, aminothiols, dithioacids, thioamides, 2-mercaptoimidazoles, thiocarbamates, mercaptopyrimidines and thiocarbamides (38). In animal models, the bioactivation of the latter two groups of xenobiotics is catalyzed primarily or exclusively by FMO (38). FMO catalyzes the sequential S-oxygenation of thioureas and 2-mercaptoimidazoles to sulphinic acids through intermediate sulphenic acids (39). The intermediate sulphenic acid is a potent thiol oxidant and the FMO-catalyzed oxidation of thiourea, phenylthiourea, ethylenethiourea and methimazole to thiol reactive metabolites (based on predicted disulphide formation) has been demonstrated both in vitro (40, 41) and in vivo (41). Because the parent xenobiotic is regenerated upon reduction, these xenobiotics establish a futile cycle catalyzing the oxidation of cellular thiols by NADPH and oxygen that can deplete cellular stores of glutathione (38). In fact, structural characterization of a glutathionyl-spironolactone disulphide arising from FMO-mediated sulphenic acid formation has been reported (42). The sulphinic acids
produced by the FMO-catalyzed oxidation of the intermediate sulphenic acids are also quite reactive and ones bearing only hydrogen or alkyl substituents on the nitrogens react nonenzymatically with free amino groups of proteins or nucleic acids forming covalent guanidine adducts (38). Often, the contribution of FMO to the metabolism of a given compound can be assessed by its unique stereoselectivity relative to other oxygenases. For example, the cytochromes P450 oxidize (S)-nicotine to a mixture of cis- and trans-N-1’-oxides. In contrast, (S)-nicotine is oxidized by human FMO3 exclusively to the trans-N-1’-oxide (38).

Aside from the toxicological implications of FMO activity, interest in these enzymes has been driven by their capacity to affect the pharmacological impact of medicinal compounds. As is the case with the P450s, variations exist in the amounts of these enzymes between different tissues and stages of development. Furthermore, these same enzyme levels appear susceptible to modulation in response to endocrine and nutritional status. The clinical and pharmacological significance of FMOs has been underlined by evidence emerging from studies of individuals apparently deficient in normal FMO activity. For example, it has been shown that the N-oxygenation of both trimethylamine (43) and nicotine (44) are subject to genetic polymorphism. The former underlies the clinical condition known as trimethylaminuria, or fish odour syndrome, and will be discussed further below.

1.2 The FMO catalytic cycle

Unique features within the catalytic cycle of FMOs are responsible for the wide range of structurally diverse molecules which can act as their substrates. The enzymic properties and catalytic mechanism of FMO were originally characterized using a homogeneous protein preparation from pig liver (12), now recognized as constituting predominantly the FMO1 isoform. Figure 1.1 outlines the major steps in the FMO catalytic cycle elucidated from this characterization. It is generally assumed that the catalytic mechanism is very similar for the other members of the FMO family, although this has not been proven so far. What has been shown, however, is that the substrate binding channels of the different FMO forms are clearly distinct (45-47). Therefore, it has been suggested that the development of functional substrate probes for the different FMO forms will be achieved through the use of molecules possessing stereoisomerism (48).
Figure 1.1. Major steps in the pig liver FMO catalytic cycle. Nucleophilic attack by the substrate (S) on the terminal oxygen of the enzyme-bound hydroperoxyflavin, followed by heterolytic cleavage of the peroxide, forms oxygenated product (SO) (1). Release of either $\text{H}_2\text{O}$ (2) or NADP$^+$ (3) is the rate limiting step in the cycle. Flavin reduction by NADPH (4) and addition of oxygen (5) complete the catalytic cycle and regenerates oxygenating hydroperoxyflavin. For more details, see text.
Significant stereoselectivity differences have already been observed for different FMO forms (47). Unfortunately, successful attempts to derive the three-dimensional structure of an FMO have not been reported.

The visible spectrum of fully oxidized FMO1 is similar to that of other flavoproteins ($\lambda_{\text{max}}$ of 445 and 380 nm and shoulder at 480 nm). A critical feature of the FMO catalytic cycle is that prior to binding of substrate or oxygen, two electrons are donated by NADPH to the isoalloxazine ring of the FAD prosthetic group to generate reduced flavin adenosine dinucleotide (FADH$_2$) (Figure 1.1, step 4). The NADP$^+$ produced apparently remains at the active site of the enzyme. At this stage, the spectrum obtained is similar to the fully oxidized flavoprotein but is shifted to a shorter wavelength ($\lambda_{\text{max}}$ 440 and 370 nm, with no apparent shoulder) (49-51). Reoxidation by molecular oxygen follows to generate the C4a-hydroperoxyflavin entity, which is stabilized by the surrounding protein microenvironment (Figure 1.1, step 5). This generates a spectrum similar to that of the flavin hydroperoxide observed with other flavoproteins (specifically, bacterial luciferase) or synthetic isoalloxazine hydroperoxides (52). It is this oxygen activated state, analogous in its oxidizing capacity to alkyl hydroperoxides, peracids, or other similar agents (53-57), that the enzyme remains in whilst not bound to substrate. The hydroperoxyflavin intermediate is unusually resistant to decomposition and remarkably long-lived (58, 59). This stability probably necessitates that non-nucleophilic amino acid residues at the active site provide a hydrophobic environment. It appears that the xenobiotic substrate interacts only with the 4a-hydroperoxyflavin form of the enzyme, becoming oxidized by oxygen transfer from this group (Figure 1.1, step 1). Oxidation of the xenobiotic substrate is a second order reaction not saturable by substrate. The oxygenated product is released immediately. There is no evidence that substrate binding lowers the energy of activation for oxygen transfer from the hydroperoxyflavin to substrate or that precise fit to substrate at more than one point on the enzyme is necessary. The lack of detectable equilibrium binding is consistent with rate constants defining the Michaelis constant ($K_m$) deduced from steady state measurements. A model consistent with all evidence currently available indicates that at saturating concentrations of xenobiotic substrates, the catalytic site of the enzyme is unoccupied most of the time. (This property may explain why non-substrate analogues of xenobiotic substrates do not inhibit FMO activity.) Thus, FMOs are capable of monooxygenating a wide range of structurally diverse compounds: nucleophilic tertiary amines, secondary amines, secondary hydroxylamines, sulphides, thiones and phosphines are all converted to their tertiary amine N-oxides, nitrones, and S- and P-oxides, respectively. Here can be
seen one of the fundamental differences between FMOs and other monooxygenases: on the basis of the chemical product produced by treating a substrate with a peracid of peroxide, the structure of an FMO metabolite can be predicted with great accuracy on most occasions. In the case of, for example, the cytochromes P450, substrate binding is required before electron transfer from cytochrome P450 reductase to the haem iron prosthetic group and binding of molecular oxygen, a requirement which retards the release of highly reactive oxygen species but also necessitates that substrates for any given P450 subfamily be similar in topology over at least a certain region of the molecule. Thus, different cytochrome P450 forms catalyze the monooxygenation of structurally related compounds. There is an analogy often quoted to describe the catalytic mechanism of FMO: ‘Unlike other oxidases, FMO sits in the cell in the loaded, cocked position and any suitable target that wanders within range will be oxidized’ (from the late Henry Kamin). Another fundamental difference between FMOs and the cytochromes P450 centres on the apparent two versus one electron mechanism of oxygenation for each monooxygenase, respectively (11). Whereas the two electron oxidation products produced by FMO have not been shown to inactivate FMOs, they have on occasions been shown capable of covalently modifying cytochromes P450 (and other proteins). For instance, sulphenic acids (38-42, 60) and sulphinic acids (36, 60-68) are, as I have already stated (Section 1.1: Introduction), reactive metabolites generated by FMO, and these two classes of chemical entity have been shown capable of inactivating cytochromes P450 and covalently labelling other proteins. This has lead to warnings concerning the interpretation of data relating to cytochrome P450 inhibition studies when the chemicals being tested are substrates for FMO (i.e. thiols, thioureas, thioamides and so forth).

It may be wondered, given the somewhat unusual catalytic mechanism of FMO, how it is that indiscriminate oxidation of physiologically essential soft nucleophiles is prevented. One must remember that the cell is rich in nucleophiles readily oxidized by organic peroxides. Although the lack of a three-dimensional FMO structure prevents an exact molecular description of how essential endogenous nucleophiles are excluded from the FMO active site, substrate structure-activity studies suggest that ionic groups on cellular nucleophiles are an important factor (18, 69). FMO readily catalyzes the oxidation of monocationic amines and anionic sulphur compounds when the charge is localized on the sulphur (for example, thioacetate), but the addition of a second charged group anywhere on the molecule tends to block substrate activity. FMOs have never been shown capable of oxidizing dianions, dications or dipolar ions, with the exception of methionine (26). Apart from cysteamine
(which is a substrate for FMO), all other essential cellular nucleophiles are
dications (polyamines), dipolar ions (amino acids and peptides) or bear one or
more anionic groups distal to the nucleophilic heteroatom (for example,
coenzyme A, biotin, thiamin and pyrophosphate). Thus, it would seem that the
position and number of ionic groups are responsible for the manner in which
FMOs discriminate between essential and xenobiotic soft nucleophiles. The
presence of charged groups is believed to have been exploited by living
systems as a way of keeping essential metabolites within the cell, whereas
uncharged xenobiotics (or those in equilibrium with their charged species)
readily cross cell membranes by passive diffusion. It would therefore be logical
for FMOs to evolve substrate specificities that exploit this.

1.3 The family of mammalian FMOs

It was originally believed that only a single form of FMO existed in mammalian
tissues. The possibility that multiple forms of FMO might exist was first
suggested by Devereux et al. (70), who observed that Hg$^{2+}$ and Mg$^{2+}$
increased the rate of dimethylaniline $N$-oxidation catalyzed by FMO partially
purified from rabbit lung, but decreased the rate mediated by a similar
preparation from rabbit liver. Analysis of the oxidation of $N^\prime,N^\prime$-dimethylaniline,
imipramine and chlorpromazine in hepatic and pulmonary microsomal
preparations from rabbit and rat reinforced the hypothesis that FMOs expressed
in different tissues of the same species might be distinct and suggested that
FMOs expressed in the same tissue of different species might exhibit unique
properties (71-73). Purification and characterization of FMO from rabbit lung
and liver provided conclusive evidence that distinctly different FMOs can be
isolated from lung and liver (74, 75). An inability of polyclonal antibodies to the
rabbit lung FMO to react with FMO purified from the rabbit liver was evidence
of significant structural dissimilarity between these two enzymes. Catalytic
differences between the two enzymes, such as the ability of the lung enzyme to
metabolize certain primary amines and of the liver FMO to metabolize
chlorpromazine and imipramine, were also found (76, 77). Furthermore, in
contrast to the FMO expressed in liver, the lung FMO was found to be more
stable when subjected to treatment with detergents or elevated temperature
(78). The lung and liver FMOs were also found to be characterized by distinctly
different pH optima (9.8 vs. 8.5). The simplest explanation for these structural
and functional differences between the liver and lung FMOs was to postulate
the existence of more than one $FMO$ gene. However, prior to 1989, no
sequence information, even for selected peptides had been published. Direct
sequencing of the FMOs was prevented by blocked N-termini and isolation of peptides from proteolytic digestions proved difficult. In the end, the structural relationship between the lung and liver FMOs was in the main defined by the use of molecular biology techniques to clone and sequence the cDNAs.

To date, the cDNAs of five different mammalian FMO enzymes have been isolated and sequenced, with some evidence for the existence of allelic variants (79, 80). After a period during which different nomenclature systems were in use for the FMOs, the potential for confusion has been eliminated by common agreement to name these five FMOs as FMO1 to FMO5, italicized for the gene and cDNA, non-italicized for the mRNA and protein (31).

cDNA oligonucleotide sequencing in particular, as well as automated Edman degradation sequence and mass spectral analysis, means that we now have substantial sequence information for the different mammalian FMOs. Ozols has made a significant contribution to the isolation, purification and Edman sequencing of three FMOs from rabbit liver (81-83). This was a notable achievement in light of the extremely lipophilic and intractable nature of highly purified FMO proteins and the presence of the aforementioned N-terminal blockade (N-acetyl group).

To date, at least twelve complete cDNA nucleotide sequences for mammalian FMOs have been submitted to the GenBank database. Generally, the sequences are 2.2-2.6kb in length.

Table 1.1 lists some of the fundamental properties of the five known human FMOs. Although each FMO will be more thoroughly discussed in turn later, it is noticeable that FMO4 stands out as being around 25 amino acid residues longer and around 3000 daltons heavier in molecular mass than the other four forms. Following cDNA sequence comparisons, it was revealed that all of the additional residues of FMO4 are located at the carboxy terminus of the protein (84). This probably came about through the loss of a termination codon in an ancestral FMO4 gene via a point mutation (84) (a similar extension is present at the carboxy terminus of rabbit FMO4 (85)). The isoelectric point (pI) values of the five proteins are all in the basic range, apart from FMO1, which is neutral.
<table>
<thead>
<tr>
<th></th>
<th>FMO1</th>
<th>FMO2</th>
<th>FMO3</th>
<th>FMO4</th>
<th>FMO5</th>
</tr>
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<tbody>
<tr>
<td>Length (No. amino acids)</td>
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<td>535</td>
<td>532</td>
<td>558</td>
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<td>Molecular mass</td>
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<td>60 903</td>
<td>60 047</td>
<td>63 338</td>
<td>60 225</td>
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<tr>
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<td>8.9</td>
<td>8.3</td>
<td>9.1</td>
<td>8.6</td>
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Table 1.1. Fundamental physical properties of human FMOs

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<th>FMO3</th>
<th>FMO4</th>
<th>FMO5</th>
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<td>83</td>
<td>84</td>
<td>85</td>
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<tr>
<td>Pig FMOs</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Rat FMOs</td>
<td>82</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>Guinea pig FMOs</td>
<td>-</td>
<td>86</td>
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Table 1.2. Percentage amino acid sequence identity between human FMOs and their known mammalian orthologues.

<table>
<thead>
<tr>
<th></th>
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<th>Human FMO2</th>
<th>Human FMO3</th>
<th>Human FMO4</th>
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<tr>
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<td>-</td>
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</tr>
<tr>
<td>Human FMO5</td>
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<td>56</td>
<td>53</td>
<td>52</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1.3. Comparison of percentage amino acid sequence identity between the five known human FMOs.
1.3i Primary structures of the mammalian FMOs

Analysis of the five FMO cDNAs reveals that they code for similar overall amino acid compositions (86). As would be expected of integral membrane proteins, there is a relatively high representation of non-polar residues (43-46%) (86). Comparison of amino acid sequences for numerous mammalian FMO enzymes has revealed that certain characteristic structural features, namely the predicted binding domains for FAD and NADPH and regions of marked hydrophobicity (hence, possible regions of membrane association), are located at very similar locations along the respective polypeptides. Indeed, computer generated hydropathy profiles (calculated by the program of Kyte and Doolittle (87)) of the mammalian FMO proteins produces plots that are almost superimposable, even between different forms (which, as I shall discuss, have only 50-60% sequence identity) or between regions of marked sequence dissimilarity (only 25-30% identity). Figure 1.2 depicts the location of structural features common to all five known rabbit FMO enzymes and probably mammalian FMOs in general. As can be seen, the Gly-Xaa-Gly-Xaa-Xaa-Gly/Ala core consensus sequences for binding of FAD and NADPH are consistently located at or near residue positions 9-14 and 186-196, respectively. These ADP-binding domains have been found in the same corresponding positions of all mammalian FMO sequences examined to date. It is thought that all mammalian FMOs bind the FAD prosthetic group at the N-terminal of the two domains and the NADPH cofactor at the other domain, based on sequence analysis (88) and site-directed mutagenesis studies of rabbit \textit{FMO2} (89). Furthermore, there are several regions of FMO sequence that appear well conserved amongst all forms, for example, residues 1-200 and between residue 450 and the C-terminus. Perhaps, therefore, these stretches represent regions of the FMO protein crucial for functional enzyme activity. Most of the FMO cDNAs sequenced to date contain the favourable Kozak translation initiation site (XXATGG, where X is generally C (90)). With the exception of FMO4, all FMOs sequenced to date have at least one putative consensus \textit{N}-glycosylation site (Asn-Xaa-Ser/Thr). Direct evidence for FMO glycosylation has come from Korsmeyer \textit{et al.}, who showed that pig FMO1 is glycosylated (91). Subsequently, it was shown by HPLC-mass spectrometry studies that a saccharide is attached to Asn 120 of this protein (92). However, the impact of such glycosylation on the function of FMO is at present unclear, given that cDNA-expressed FMO1 and FMO3 in \textit{E.coli} results in no detectable change in substrate stereoselectivity (46, 47, 93).
Figure 1.2. Common structural features of the five known FMO enzymes (FMO1-5) of rabbit. Shaded areas represent hydrophobic regions of amino acid sequence predicted by computer software to have membrane-association potential. The position of the predicted FAD and NADPH-pyrophosphate-binding domains are indicated. Underlined sequences are those found in each of the five rabbit FMO sequences. Regions characterized by marked dissimilarity between the five sequences are indicated by 'xxxxx'. Examination of other mammalian FMO amino acid sequences reveals that the relative positions of the FAD and NADPH-pyrophosphate-binding domains, and the locations of marked regions of hydrophobicity, are virtually identical.
Amino acid sequence identities between orthologous FMO forms from different mammalian species are consistently in the 82-88% range (31, 86). Table 1.2 lists a comparison of primary structures between human FMOs and those that are known for rabbit, pig, rat and guinea pig. By contrast, comparison of sequence identity between different FMO forms from the same mammalian species reveals a sequence identity of just 51-57% (31, 86) (Table 1.3).

The comparative sequence data permit speculation on the evolutionary events that gave rise to the present-day mammalian FMO gene family. The sequence identity between orthologous FMO forms from different mammalian species is around 30% greater than it is between different FMO forms from the same species. Also, the degree of sequence identity between any two of the five known FMO forms from the same mammalian species appears relatively consistent.

It would appear from this that repeated duplication of a common FMO ancestral gene took place over a relatively short period of time prior to mammalian radiation, an event thought to have occurred some 85 million years ago. Over the period of these 85 million years since mammalian radiation, orthologous FMO enzymes have diverged in sequence by an average of around 15%. This allows us to calculate the unit evolutionary period (UEP), defined as the time taken for a 1% divergence of sequence, for these enzymes. The UEP for mammalian FMOs turns out to be close to five million years. By way of comparison, the UEP for the cytochromes P450 averages at about three million years (94). If we make the assumption that the UEP of five million years for FMOs has remained relatively constant since the postulated duplication of the common ancestral gene, then the 52-57% sequence identity that exists between different FMO forms allows us to estimate when this genetic event took place. It emerges that the time taken for 45% of the sequence to diverge between the respective FMO forms calculates to about 290 million years. If this is an accurate estimate, it would suggest that the mammalian FMO gene family we observe today was initially formed at a period very close to the Carboniferous-Permian border in evolutionary history (an estimated 286 million years ago). At this point in evolutionary history, it is believed the first primitive reptiles evolved from amphibians (the first vertebrates to move from sea to land), which in turn were to eventually give rise to the dinosaurs, birds, lizards, snakes, crocodiles, marsupials and mammals. It is interesting to speculate why the additional FMO genes formed by duplication of the common ancestral gene have been maintained from this point in evolution onwards, rather than decaying to form inactive pseudogenes, as would occur if there were no
selective pressure to maintain their ability to code for active enzymes (it is, of course, entirely possible that the duplication events gave rise to more than the five known FMO genes, or however many active FMO genes there are, but were of no selective advantage and became pseudogenes; such pseudogenes may yet be discovered). Indeed, it would appear from recently published work that in most humans the FMO2 gene does not produce a functional protein and is, therefore, a pseudogene (80) (this finding is discussed at length later). Perhaps the answer lies in the fact that at the postulated time of the duplications, vertebrates were beginning to colonize dry land for the first time and new species were rapidly evolving to exploit the huge number of available niches. This will have presented to these organisms the need to cope with exposure to a myriad of new xenobiotics, for example, the almost limitless number of chemicals ingested from the consumption of new plant materials. It should not be forgotten just how large is the range of chemicals found within foodstuffs, particularly vegetation. Aside from the nutritive proteins, fats, carbohydrates, vitamins, minerals and so forth, there are many xenobiotic substances that have no nutritional value. The structural nature of these compounds is diverse indeed: phenolic substances, steroids, terpenes, flavins, coumarins, xanthines, glycosides, to name but a few. (95). Additional FMO genes that could evolve different substrate specificities would almost certainly have given their hosts a selective advantage during this crucial and spectacular stage of vertebrate evolution on land.

Rabbit FMO1, FMO2 and FMO5 sequences have each been shown to bear a consensus sequence common to serine proteases and esterases (83). Specifically, Ser-194 within the sequence Gly-Asn-Ser-Gly-Thr is a conserved feature of each of these three enzymes that has homology to the active site serine residue (Ser 195) in serine proteases and esterases. In addition, the FMO3 sequence has the consensus sequence containing the histidinyl residue of the catalytic triad of the diisopropylphosphoryl-sensitive carboxylesterases and thioesterases. Because of this, it has been suggested that the common ancestral FMO gene may itself have evolved from the serine proteases.

I will now consider in more detail features of each of the five known FMO enzymes and their respective genes, drawing comparisons between the forms where appropriate.

1.3ii FMO1
Purification of FMO1 to homogeneity from pig liver provided the first unambiguous evidence for a heteroatom N- or S-oxygenase (96). Isolation was
achieved by extraction of microsomes with detergents, selective fractionation, and purification with column chromatography on ion-exchange resins. The enzyme was shown to be devoid of cytochromes, haem or metals, to contain 15.1-15.3 nmol of FAD/mg protein and to catalyze the NADPH-dependent oxygenation of prototypic substrates such as dimethylaniline or methimazole (18) (subsequently, it was shown that highly purified FMO1 contains variable amounts of lipid (97)). One property that distinguished this FMO enzyme from other monooxygenases known at the time was its unusual thermal lability. Rapid chilling of the pig liver (hepatic tissue temperature can rise quickly after death) was required if FMO activity was to be retained. Indeed, in the absence of NADPH, approximately 85% of the activity of pig liver FMO was lost after incubation at 40-45°C for 3-5 min, conditions not unlike those encountered under post-mortem conditions (18). Lipid alkyl hydroperoxides probably have a negative effect on FMO1 enzyme action and stability, whereas the addition of antioxidants such as butylated hydroxytoluene apparently assist in enzyme stability and purification. Julius Ozols in 1989 (98) demonstrated conclusively through peptide sequencing the existence of two distinct FMO enzymes in rabbit liver, one of which was FMO1, the other FMO3. A year later Ozols published the full amino acid sequence of the FMO1 enzyme, again using peptide sequencing techniques (81), to reveal a hydrophobic carboxy-terminus and an N-acetylated alanine at the amino-terminus. Soon afterwards, Gasser et al. published details of the isolation and sequencing of the full-length FMO1 cDNA from pig liver (99). The sequence predicted a 532 amino acid protein of molecular mass approximately 59kD with a classic FAD binding domain (Gly-Xaa-Gly-Xaa-Xaa-Gly) at positions 9-14. When sequence comparison was made with the rabbit FMO1 sequence derived by Ozols, it was realised that both enzymes were orthologous isoforms. Western blot analysis of rabbit lung and kidney microsomal samples with antibodies to the purified pig liver FMO revealed cross-reactivity with proteins of identical electrophoretic mobility, thereby suggesting that in the rabbit, FMO1 was also expressed in extra-hepatic tissues. Confirmation of this was provided via northern analysis with the rabbit liver FMO1 cDNA as probe. Hybridization was seen to take place to mRNA species from rabbit liver, lung and kidney. In the same year, Lawton et al. published the full-length cDNA sequence for rabbit FMO1 (100). From this, the corresponding protein was predicted to be 535 amino acid residues in length (equivalent to a molecular mass of 59.8kDa) and to have 87% sequence identity to the pig FMO1 sequence.

Evidence of immunological differences between FMO1 forms from different species exists. Anti-mouse liver FMO antibodies do not recognize pig liver FMO
and antibodies raised to pig liver FMO do not cross-react with purified mouse liver FMO (101). Furthermore, western blot analysis of microsomal fractions from mice, rats, rabbits, dogs and humans shows that clear species differences exist in the amounts of hepatic FMO (102, 103). Note: the anti-pig liver FMO serum used by Dannan and Guengerich (102) was apparently a unique serum possessing unusual antigen-recognition properties. Immunoblot studies with other anti-pig liver FMO sera has not produced such pronounced results for species differences in hepatic FMO levels.

It was not until 1991 that work was published concerning the characterization of human FMO forms. Dolphin et al. (104) published details of the isolation and subsequent sequencing of a full-length cDNA from a human hepatic cDNA library using pig hepatic FMO1 cDNA as a probe. As might be expected, the predicted amino acid sequence from the cDNA possessed a high degree of sequence similarity with previously published pig FMO1 and rabbit FMO1 cDNA sequences (88 and 86%, respectively) but less identity with the rabbit FMO2 cDNA sequence (56%). Furthermore, it was found that the putative FAD and NADP binding sites within the human FMO1 sequence aligned with those identified in the pig and rabbit FMO1 sequences. A hydropathy profile of the protein sequence revealed the presence of hydrophobic regions of 15-20 amino acid residues at both the amino- and carboxy-termini. The former region encompassed the proposed FAD binding site and preceded a predicted β-turn-β-turn-α-helix region of secondary structure termed the ‘Rossmann fold’, a structure known to be involved with dinucleotide binding (105). The hydropathy plot further revealed another three hydrophobic segments at positions 138-150, 322-338 and 366-380. None of these hydrophobic regions, when analyzed, were predicted to form the α-helical or β-sheet secondary structures believed typical of membrane spanning domains (106) and so a region of the protein involved in its anchorage to lipid membranes could not be postulated. The putative NADP binding site (residues 191-196) was found to exist between two regions that were predicted to adopt β-sheet and α-helical conformations, respectively. Northern analysis revealed that, in contrast to the pig and rabbit, human FMO1 mRNA is very poorly represented in adult liver. In contrast, however, northern hybridization analysis of foetal liver samples showed that, at this point in human development, FMO1 mRNA is relatively abundant. Such a finding was in agreement with the much earlier work of Rane (107), who found N,N'-dimethylaniline N-oxygenase activity characteristic of pig FMO1 in human foetal microsomes. Southern blot hybridization of human genomic DNA indicated that human FMO1 is encoded by a single gene. The genomic location of this gene, the first FMO gene for which this was
successfully achieved, was identified through the use of the polymerase chain reaction to screen human-rodent somatic cell hybrids. This conclusively identified the location of the human FMO1 gene to be chromosome 1 (104).

Recently, genomic stretches of the rabbit FMO1 gene have been isolated (108). Genomic DNA as opposed to the cDNA, allows the intron-exon structure of the gene to be elucidated and may yield some 5' flanking promoter sequences. The isolation of the promoter sequences are essential if the process of understanding the molecular basis of regulation of FMO gene transcription is to begin. Wyatt et al. screened a rabbit genomic DNA library constructed in lambda FIXII vector with rabbit FMO cDNA sequences. Seven clones were isolated and established to encompass part of the FMO1 gene. Overlapping just three of these clones was sufficient to span the entire gene, which was estimated to be about 40kb in size. Further analysis of these three clones demonstrated that the entire rabbit FMO1 gene consists of nine exons and eight introns. Exon 9 contained both the 3' end of the coding sequence and all of the 3'-noncoding region of the corresponding mRNA. A putative polyadenylation signal, AATAAA, was identified twelve nucleotides beyond the end of the published cDNA sequence. Exon 1 was found to be entirely 5' noncoding sequence, with the translational start codon located close to the 5' end of exon 2. The use of the ribonuclease protection assay and 5' rapid amplification of cDNA ends (5' RACE) indicated that the gene for rabbit FMO1 has multiple putative transcription start sites. Furthermore, within the 5' flanking region of the gene there was located a consensus glucocorticoid response element: this suggested steroid-induced enhanced levels of expression for this gene, of which there was already evidence (109, 110) (see Section 1.4: FMO activity in relation to endocrine and nutritional status).

I.3iii FMO2

FMO2 was the second mammalian FMO enzyme identified after FMO1. As has been mentioned already in this Section, from the 1970s onwards there was accumulating evidence suggesting that the ‘liver’ FMO form was not the only mammalian FMO isoform in existence and that another distinct FMO isoform was present in lung tissue. As a consequence, there is an abundance of comparative data between these two enzymes. This includes the observation that FMO activity from rabbit hepatic microsomes (FMO1) possesses immunochemical and enzymological properties distinct from FMO activity in rabbit lung microsomes (FMO2) (75, 78, 103, 111). It was subsequently shown that the ‘lung’ FMO form was present in especially high concentrations in rabbit foetal lung (perhaps suggesting a role in the metabolism of agents traversing the
placental membrane) and that it could also be detected in rabbit kidney and bladder. Comparison of the enzymological properties of pig FMO1 and rabbit FMO2 have shown that substrate specificity (74, 75) and substrate stereoselectivity (112-114) are quite distinct between these two enzymes, although some classes of substrates are efficiently oxygenated by both. For example, FMO1 and FMO2 both efficiently N-oxygenate dimethylaniline and S-oxygenate thiobenzamide and thiourea (6, 45). Rabbit FMO2 N-oxygenates long, aliphatic primary amines, whereas FMO1 does not (45), although FMO1 sometimes utilizes aliphatic primary amines as positive effectors of enzymic action. Alternatively, short side-chain tertiary amines (such as the tricyclic antidepressants chlorpromazine and imipramine, and other [(N,N-dimethylamino)alkyl]phenothiazine derivatives) are readily oxygenated by FMO1 but not FMO2 (73). As a result, it has been suggested that the substrate binding channels for FMO1 and FMO2 are quite distinct and that FMO2 requires a nucleophilic nitrogen centre possessing at least a C6 or C7 alkyl side chain (45). Furthermore, it has been proposed that larger substrates are admitted to within 3Å of the enzyme hydroperoxyflavin in the case of pig FMO1 and 6-8Å in the case of rabbit FMO2, with the binding site channel spanning as much as 12Å in diameter for pig FMO1 and only 8Å in the case of rabbit FMO2 (48).

FMO1 and FMO2 stereoselectivity studies of various S- and N-containing substrates have largely confirmed the above hypothesis for FMO binding channel dimensions. Sulphur-containing substrates such as 4-bromophenyl-1,3-oxathioline (93, 115, 116), aryl-1,3-dithiolanes (113, 117), and 2-methyl-1,3-benzodithiole (112) show an almost exclusive preference for rabbit FMO2-mediated S-oxygenation of one sulphur atom lone pair. By contrast, although pig FMO1 displayed marked stereoselectivity (118), in general it did not show the absolute stereoselectivity observed for rabbit FMO2. Stereoselective N-1’-oxygenation of (S)-nicotine by FMO1 and FMO2 provides further evidence that the binding channels of both monoxygenases are distinct. FMO2 forms exclusively trans-(S)-nicotine N-1’-oxide (119), and FMO1 forms approximately a 1:1 mixture of cis:trans-(S)-nicotine N-1’-oxide (120-122). FMO1 and FMO2 also show distinct thermal lability (18, 78). In the absence of NADPH, rabbit FMO2 is stable at 45°C for 10 minutes, whereas pig FMO1 remains active for only 5 minutes at this temperature. Another difference is that FMO2, but not FMO1, is resistant to inactivation by anionic detergents, which impacts on the chromatographic methods used to isolate these two enzymes (75, 78, 96). Incidentally, low concentrations of bile salts, such as cholate, appear to stimulate FMO activity in mouse and rat liver preparations (1, 123-125) but decrease FMO activity in hepatic preparations from rabbit (126) and pig (78). Mercury
stimulates rabbit FMO2 (70, 109) but decreases rabbit FMO1 activity (70, 123). Differences are also observed in response to magnesium (70). Rabbit and mouse FMO2 have optimal enzyme activity at approximately pH 10 (75, 78, 127), while for pig FMO1 the optimum pH is around 8.3-8.5 (18) and for mouse liver FMO it is 8.8-9.2 (101, 127). However, it is not clear whether the pH optima measured for FMO in various preparations take into account the pH dependence for ionization of many substrates utilized for the monooxygenase.

It was in 1990 that Lawton et al. published the first full-length cDNA nucleotide sequence for a mammalian FMO2 enzyme (100). The sequence was for rabbit FMO2 and comparison was made to the full-length cDNA sequence for rabbit FMO1 (100) and pig FMO1 (99). Rabbit FMO2 was a predicted 535 amino acid residues in length (molecular mass 61.1kDa) and possessed 56% sequence identity to the rabbit FMO1 form. Comparison of the positions of the putative FAD pyrophosphate and NADP pyrophosphate binding domains of pig FMO1, rabbit FMO1 and rabbit FMO2 revealed them to be located at identical sites in the respective proteins. The putative FAD pyrophosphate binding site of rabbit FMO2 contained the expected highly conserved consensus sequence (Gly-Xaa-Gly-Xaa-Xaa-Gly) beginning at residue 9. Similarly, the putative NADP pyrophosphate site of rabbit FMO2 contained the same consensus sequence beginning at residue 191. Calculated hydropathy indices revealed, as might be expected, that rabbit and pig FMO1 have almost identical indices. However, less expected was the finding that the hydropathy indices between the rabbit FMO1 and rabbit FMO2 were practically superimposable. From these indices it can be seen that rabbit FMO1 and rabbit FMO2 have in common five highly hydrophobic regions, some of which are presumably involved in lipid membrane attachment. Northern hybridization analysis of mRNA from rabbit liver, lung and kidney tissue was performed using either rabbit FMO1 or rabbit FMO2 full-length cDNAs as probes. The FMO1 probe hybridized to single (2.6kb) bands in the case of the hepatic and renal samples but did not hybridize to any mRNA species from the pulmonary preparation. The FMO2 probe, however, failed to hybridize to any mRNA species from the hepatic preparation but hybridized to four distinct bands (2.4, 2.6, 4.8 and 6.0kb) in the case of the renal and pulmonary preparations. Repeated hybridization at different temperatures demonstrated that the four bands had equal affinity for the FMO2 probe. This, combined with the results of probing the same blots with portions of the FMO2 probe, lead Lawton et al. to suggest that each of the four bands represent FMO2 mRNA in differing stages of processing or alternative splicing, rather than being the result of cross-hybridization to similar but distinct mRNAs (100). The existence of multiple
polyadenylation sites within the 3’ non-coding cDNA nucleotide sequence of \textit{FMO2} adds support to this theory. It was at first suspected that the multiple FMO2 mRNA species observed may relate to the multiple protein bands seen following western blotting of pulmonary microsome preparations and probing with antibodies raised against FMO expressed in lung. The same pattern of mRNA and protein bands is seen also to occur for the rabbit kidney (103). However, this possibility was dismissed upon finding, in the case of the guinea pig, that although the multiple protein bands also existed in lung of this species there was only a single mRNA band evident on northern analysis (100, 103). A more likely explanation, at least in part, for the multiple protein bands observed for the FMO2 enzyme would therefore be a combination of the existence of allelic variants and variations in post-translational modification. Southern hybridization analysis of rabbit genomic DNA, previously subjected to digestion with a range of restriction enzymes, was performed using a combination of full- and partial-length cDNA probes for either rabbit \textit{FMO1} or rabbit \textit{FMO2}. This indicated that both of these genes exist as single copies within the rabbit genome. Further work published by the same group soon afterwards suggested that rabbit lung and liver microsomes contain more than one form of FMO enzyme, thereby implying the existence of at least one more FMO enzyme form in addition to FMO1 and FMO2. Specifically, the kinetics of methimazole oxidation by microsomes from these tissues was biphasic in nature and contrasted to that of FMO1 and FMO2 expressed in COS-1 cells (128).

Rabbit FMO2 isolated from lung and subjected to tandem mass spectrometry and gas phase sequencing studies has provided direct evidence for the existence of a 1:1 complex with a calcium binding protein, suggested to be calreticulin (48). An FMO2-calreticulin complex might account for some of the more atypical properties of this FMO form. For example, when complexed to calreticulin, FMO2 is relatively hydrophilic. FMO2 alone reverts to a more lipophilic protein with similar physical-chemical properties to FMO1 (48). It might be that the binding of calreticulin is responsible for the greater stability of FMO2 at higher temperatures compared to FMO1, and for the non-requirement of high concentrations of detergent for FMO2 activity in contrast to FMO1. Attempts at combining calreticulin with pig FMO1 have not resulted in any observed changes in physiochemical properties. It is worth mentioning the fact that there are some discrepancies between reported rabbit FMO2 cDNA sequences and the primary structures they predict, and amino acid compositions determined directly from purified enzyme. This could be the result of some FMO2 preparations containing significant quantities of calreticulin complexed to the FMO: some FMO preparations have been found to contain equimolar
amounts of enzyme and calreticulin (129, 130). Suggested physiological roles for calreticulin have included that it may be a 'marker' for the cell, indicating that the protein to which it is bound is destined for retention within the endoplasmic reticulum. This suggestion is due to the finding that the carboxy-terminal sequence of calreticulin is found in several resident luminal endoplasmic reticulum proteins (131). Thus, aside from any possible modulation of enzyme activity, calreticulin may serve as a chaperon, targeting rabbit FMO2 to the endoplasmic reticulum.

Recently, an important and interesting finding has been reported by Dolphin et al. following study of the human FMO2 cDNA nucleotide sequence (80). It was discovered that in eight unrelated Caucasians, an in-frame translation termination codon, TAG, exists within the FMO2 nucleotide sequence at codon 472 (64 codons upstream of the expected end of the open reading frame). This was found to align with a CAG codon (glutamine-472) in the rabbit, gorilla, chimpanzee, rhesus monkey and guinea pig FMO2 sequences. Analysis of FMO2 sequence in this region from several different ethnic groups showed that the frequency of the 472-stop allele was 100%, apart from a group of African origin, which contained some individuals that were heterozygous for the 472-stop and 472-Gln alleles. Thus, it would appear that the majority of humans do not express active FMO2, assuming that the loss of 64 amino acid residues from the carboxy terminus of the protein abolishes catalytic activity. Furthermore, it was found that examination of the Kozak region sequence surrounding the translation initiation codon of the human FMO2 cDNA revealed a sequence (GAGCTGATGG - start codon in bold) with low similarity to the consensus sequence for this region (GCCA/GCCATGG). As the Kozak consensus sequence is known to be connected with efficient ribosome binding and translation initiation of vertebrate mRNAs, the poor conformity of the FMO2 sequence is probably significant. Also, the presence of a pyrimidine nucleotide within the Kozak region at the -3 position relative to the 'A' of the start codon is only rarely associated with a functional initiation codon. On the face of it, such findings would fit neatly with the hypothesis that, in humans at least, active FMO2 at one point became no longer advantageous to survival and health. As a result, the FMO2 gene ceased to be maintained by natural selection in a form capable of producing active FMO2 enzyme and the consequent decay of the gene sequence over the passage of time led to the 472-stop codon and the redundant Kozak region sequence.
The first sequence data for a mammalian FMO3 was published in 1991 by Ozols (82) in the form of the complete amino acid sequence of the rabbit enzyme. Ozols achieved this through the use of peptide sequencing techniques similar to those he used to sequence rabbit FMO1 (82), revealing rabbit FMO3 to be 533 amino acid residues in length and of approximate molecular mass 60kD. In common with the other FMO forms sequenced to date, the carboxy terminus of rabbit FMO3 was found to be distinctly hydrophobic and the FAD and NADP binding domains located in corresponding positions to other FMO forms (i.e. around residues 9 and 190, respectively). The sequence identity with rabbit FMO1 and the rabbit FMO2 was 52 and 55%, respectively. Of possible interest with respect to locating the site of substrate binding was the identification of three areas of sequence variability: residues 308 to 321, residues 408 to 421 and the hydrophobic carboxy terminus (residues 505 to 533). Rabbit FMO3 is apparently inherently less stable than rabbit FMO1 and more susceptible to proteolytic attack. Ozols proposed that this could be due to FMO1 from both rabbit and hog possessing an N-acetyl alanine residue at the amino terminus, whereas FMO3 begins with a free glycine residue; it has been proposed that N-acetylation may affect the biological function and/or stability of proteins.

The full-length human FMO3 cDNA sequence was published in 1991 by Lomri et al., following screening of a human adult liver cDNA library (132). It revealed the corresponding protein to be 533 amino acid residues in length and to have a predicted mass of approximately 59kD. The predicted amino acid sequence of FMO3 was calculated to be 80% identical to that of rabbit FMO3 (comparison to the Ozols sequence (82)) but only 52, 55 and 53% identical to rabbit, pig and human FMO1, respectively. Subsequent to this publication however, Dolphin et al. have published the nucleotide sequence of an independently isolated human FMO3 cDNA and found it to be at variance with the sequence published by Lomri et al. (133). The predicted FMO3 amino acid sequence of Dolphin et al. differs from that of Lomri et al. at seventeen residues when the two sequences are aligned. (Note: Dolphin et al. have very recently communicated that further studies on FMO3 have revealed that one of these seventeen differences may in fact not so: apparently, the Lomri prediction that there is isoleucine at residue 486, in common with the equivalent position in all other FMO sequences reported to date, is almost certainly correct and not methionine as originally reported by Dolphin et al. (134)). Furthermore, alignment between the two respective amino acid sequences requires the introduction of three single-residue gaps, and the protein predicted by Dolphin et al. is one residue shorter at 532 amino acids. Numerous discrepancies were also reported in the
respective 5' and 3' non-coding regions. The revised sequence of Dolphin et al. has a predicted amino acid sequence sharing 84% identity with that of the predicted rabbit FMO3 amino acid sequence as determined by Burnett et al. from the cDNA nucleotide sequence (85). This contrasts with the lower than expected identity of 80% between the human FMO3 amino acid sequence predicted by Lomri et al. and the rabbit FMO3 protein sequence determined by Ozols (82), although the latter sequence differed from the predicted rabbit FMO3 amino acid sequence of Burnett et al. (85) at fourteen positions. The predicted human FMO3 amino acid sequence of Dolphin et al. contains the expected two consensus sequences associated with the occurrence of βαβ-fold secondary structures ('Rossman folds') at residues 4-32 and 186-213, which are associated with binding domains for the ADP moiety of dinucleotide cofactors (105). Subsequently (134), the structural organization of the human FMO3 gene has been determined by exon to exon PCR of human genomic DNA and a novel application of the vectorette PCR technique (135). The results demonstrate that the human FMO3 gene contains nine exons, the first of which is entirely non-coding, and that the gene has a minimum size of 22.5kb. The translation initiation codon was found to be located in exon 2 and the translation stop codon in exon 9. Some 400bp of immediate 5' flanking sequence was elucidated as a result of this work. Analysis of this sequence revealed three TATA box-like sequences, an AT-rich sequence, TAATAT, and two AATAAAA hexamers. Examination of the 3' untranslated sequence of the mRNA within exon 9 confirmed the presence of two potential polyadenylation signal sequences previously identified from the cDNA sequence (132). Poly(A) addition is reported to occur approximately fifteen nucleotides downstream of the more 3' of these sequences (132). Although the sequence YGTGTYY, commonly located downstream of poly(A) addition sites (136) was not found from the gene sequence, a G/T cluster (TTTGTGTGTGT) that is associated with efficient cleavage and polyadenylation (137) was located approximately 85bp downstream of the proposed poly(A) addition site. The FAD prosthetic group binding domain was found to be encoded entirely within exon 2 and the NADP binding domain almost entirely within exon 5. Overall, the intron/exon arrangement described by Dolphin et al. for human FMO3 was noted for its strong similarity to that reported for rabbit FMO1 (108). Both genes comprise nine exons, have entirely non-coding sequences within exon 1, and in each case their entire 3' untranslated regions are located within exon 9. Additionally, the translation initiation codon is located at the same relative position within exon 2 of both genes. The evident structural conservation witnessed here only serves to reinforce the assumption that the mammalian FMO gene family arose from a common ancestral gene.
Finally, analysis of adult human liver mRNA has shown there to be only one human FMO3 mRNA species and analysis of genomic DNA demonstrates that human FMO3 is the product of a single gene (132).

1.3v FMO4
In 1992, the full-length cDNA sequence of human FMO4 (at the time, this FMO was actually named FMO2) was published by Dolphin et al. (84) using techniques similar to those used by this group to isolate and sequence the human FMO1 cDNA (104). The predicted polypeptide encoded by the cDNA is 558 amino acid residues long with a calculated molecular mass of approximately 63kD. The amino acid sequence was found to be 52% identical to that of human FMO1, rabbit FMO2 and rabbit FMO3. As is typical of all the mammalian FMOs sequenced to date, the C-terminus region of FMO4 is distinctly hydrophobic in nature, leading to speculation that this region of the enzyme is anchored to lipid membranes, despite the fact that it is not predicted to form the α-helical or β-sheet secondary structures thought typical of membrane-spanning domains (104, 106). The proposition that this part of the FMO sequence is responsible for membrane anchorage was also put forward by Ozols after establishing the amino acid sequence of rabbit FMO1 (81). However, it can probably be ruled out by the subsequent finding that rabbit FMO2, when expressed in *Escherichia coli* following deletion of the putative membrane-anchoring peptide (the 26 C-terminal amino acid residues), remains associated with the bacterial lipid membranes in a manner analogous to the intact FMO2 enzyme (89). Those N-terminal sequences of FMOs that have been subjected to hydropathy profiles also seem to be consistently hydrophobic in nature and so this region of the protein has also been put forward as a candidate for membrane association, although once more the secondary structure predictions do not yield conformations typical of membrane spanning regions. Furthermore, the N-terminus of FMOs lacks any clearly discernible signal peptide sequence and is the location of the suggested FAD-binding region (89). Comparison of the human FMO4 protein sequence with that of human FMO1, rabbit FMO2 and rabbit FMO3 revealed two areas of relatively high conservation at positions 1 to 230 and 450 to 500 (a total of 34% of all amino acid residues are absolutely conserved between all four of these sequences and of these, 70% are located within these two regions), indicating that these regions of the FMO enzyme may have important functional/structural roles (the former region includes the FAD and NADP binding domains). However, although the region of residues between 230 and 450 were found to be less conserved between non-orthologous FMOs than the two regions just described (23% of amino acid residues conserved across all four peptides), this region is conserved between
FMO1 forms, perhaps suggesting that it confers properties unique to that particular FMO form. In addition, despite the apparent lack of sequence conservation in this region between non-orthologous FMO forms, the resultant hydropathy profiles generated by these divergent sequences appear very similar in each case. The increased length of human FMO4 of about 25 amino acid residues may be accounted for by a mutation in the third base of an ancestral stop codon, allowing translation to continue through to the next in-frame stop codon at position 559, thereby generating extra amino acid residues at the C-terminus (84). As was stated previously (this Section, Subsection 1.3i: Primary structures of the mammalian FMOs), a unique feature of human FMO4 appears to be the lack of a consensus N-glycosylation sequence (Asn-Xaa-Thr/Ser); all other FMO sequences reported to date contain at least one potential oligosaccharide acceptor sequence (pig FMO1 has been demonstrated to be glycosylated at one or both of its N-glycosylation sites). Human FMO4, as well as human FMO1, rabbit FMO1, pig FMO1 and rabbit FMO2, has methionine as the N-terminal residue followed by an alanine residue. It has been shown that N-terminal sequences such as these promote post-translational modification leading to the removal of the methionine residue followed by acetylation of the following alanine residue (138). Indeed, peptide sequencing has revealed that the N-terminal methionine residue is absent from mature forms of pig FMO1, rabbit FMO1 and rabbit FMO2 (81, 129, 139). The results of northern blot hybridization analysis indicate that human FMO4 mRNA is present in low abundance in adult human liver (nevertheless, in amounts far higher than FMO1, the form originally termed the ‘hepatic’ FMO enzyme due to it being the predominant FMO enzyme in rabbit and pig liver), and also in lung and kidney (84). Unlike human FMO1, however, human FMO4 is not present in greater abundance in foetal liver (84). Southern analysis of genomic DNA with single exon probes established that human FMO4 is the product of a single gene (84).

1.3vi FMO5

The first mammalian FMO5 to be identified and characterized was that of rabbit (140). After initial screening of rabbit liver cDNA libraries at low stringency with rabbit FMO1 and FMO2 cDNAs, Atta Asafo Adjei and coworkers eventually isolated a full-length (2.2kb) cDNA encoding a protein of 533 amino acid residues, which, it soon became apparent, was a new and fifth mammalian FMO form. The rabbit FMO5 amino sequence was determined to be 52, 57 and 55% identical to the rabbit FMO1, FMO2 and FMO3 amino acid sequences, respectively, and contained the characteristic two putative pyrophosphate binding domains and hydrophobic carboxy terminus characteristic of FMOs.
Northern analysis using the rabbit FMO5 cDNA as a probe revealed two species of mRNA in the rabbit liver and kidney (2.6kb and 5.4kb) but not lung. Similar analysis of other species revealed that guinea pig, hamster, rat and mouse express FMO5 mRNA in liver, kidney and lung. Expression of the rabbit FMO5 cDNA in *Escherichia coli* followed by purification revealed that it could not metabolize the general FMO substrate methimazole, although it was highly active with n-octylamine. Comparison of its temperature lability, responses to ions and detergent, and pH optimum with that of rabbit FMO1 revealed little in the way of significant differences.

Human FMO5 characterization was not reported until 1995, when Overby and coworkers (141) published their results following the successful screening of human and guinea pig hepatic cDNA libraries with the cDNA encoding rabbit FMO5. The human and guinea pig cDNAs both encoded proteins of 533 amino acid residues containing the expected putative pyrophosphate binding domains. The predicted amino acid sequences for human and guinea pig FMO5 were found to be 87% identical. When the comparison was made between guinea pig FMO5 amino acid sequence and rabbit FMO5 amino acid sequence the similarity was 85%; between human FMO5 and rabbit FMO5 it was 82%. The cDNA-derived sequences for human, guinea pig and rabbit FMO5 were noted to differ in length by nineteen residues from the reported peptide sequence reported by Ozols for FMO5 purified from rabbit liver (83). This difference was accounted for by the cDNA-derived sequences being twenty residues longer at the carboxy terminus and two residues longer at the amino terminus, and by an insertion of three amino acids at position 304 of the derived sequence in the Ozols sequence. In common with findings for many of the other mammalian FMOs, FMO5 in human and guinea pig was found to be encoded by multiple transcripts. The picture in this regard was more complicated for the guinea pig, which showed evidence of both 5' and 3' alternative processing of the mRNA. The variability at the 3' end might be the result of multiple 3' cleavage signals, whereas the origin of two 5' variants is unexplained, as an insertion sequence was not detected as in the case of variant 5' transcripts of FMO4 (85). Using FMO5 antibodies generated by injection of purified rabbit FMO5 enzyme (expressed in *Escherichia coli*) into goat, FMO5 was detected in samples from adult human liver, foetal human liver, guinea pig liver and rabbit liver.

Expression of both human and guinea pig FMO5 cDNAs in *E. coli*, followed by purification, has allowed further characterization of these enzymes. As with rabbit FMO5 (140), both guinea pig and human FMO5 failed to catalyze
metabolism of the general FMO substrate methimazole but were able to metabolize n-octylamine, a substrate previously thought to be specific for FMO2. Thus, FMO5 appears to possess unique substrate specificities in comparison with the other FMO forms. For instance, although the kinetics of FMO1, FMO2 and FMO3 can vary significantly with some substrates (85, 128), their specificities differ only somewhat: FMO1 and FMO3 metabolize tricyclic antidepressants (78, 140) and FMO2 metabolizes short chain amines (76, 77). Otherwise, all three forms share the same list of substrates. FMO5 activity, in contrast, appears only to occur with a few low molecular weight primary amines (140). Response of human and guinea pig FMO5 to detergent (sodium cholate), ions (MgCl2) and elevated temperature was found to be comparable to that previously witnessed for rabbit FMO5. The conclusion drawn is that, probably across all mammalian species, FMO5 appears not to be a drug-metabolizing enzyme and may therefore have some hitherto unknown physiological function. It is interesting that human FMO5 appears to be the only FMO isoform expressed in both adult and foetal liver. Human FMO1, for example, is apparently expressed only in foetal liver (104) and human FMO3 only in adult liver (86, 132, 133). One role that has been suggested for FMO5 is the regulation of prenylated proteins (142).

1.4 FMO activity in relation to endocrine and nutritional status

It has been known for some time that FMO activities are generally highest in the liver and that expression of FMO activity in certain tissues is under the influence of the endocrine (143) and nutritional status (144) of the animal. Thus, FMO activity and/or amounts have been demonstrated to alter as a function of sex, age, oestrus cycle and pregnancy, and to be influenced by dietary conditions. However, FMO is not inducible by the classical cytochrome P450 inducers, such as phenobarbital, polycyclic aromatic hydrocarbons, ethanol or macrolide antibiotics (145). Ascorbic acid-deficient guinea pigs reportedly have only 55% of hepatic FMO activity compared to ascorbic acid-adequate animals (144) and guinea pigs fed a calorie-restricted diet (leading to 10-15% weight loss) show a 77% increase in hepatic FMO activity, as measured by the rate of N-oxidation of N,N'-dimethylaniline (146). Clearly, findings such as this, if similar in humans, would suggest that the nutritional state of individuals may have a bearing on the metabolism of drugs and pollutants. Dietary xenobiotics appear to influence FMO activity in the rat liver, as animals maintained on total parenteral nutrition for seven days (i.e. on a diet completely lacking in xenobiotics) show a 75-80% decrease in hepatic FMO activity (147). Similar
studies, in which rats were fed a synthetic diet for 1 week, showed a significant drop in FMO activity, as measured by the rate of ethyl-methyl-sulphide S-oxygenation (148) and trimethylamine N-oxygenation (149) (two selective functional markers for FMO activity). Results such as this raise interesting questions as to the impact of dietary constituents on hepatic FMO activity. Ziegler and colleagues have suggested that one or more organic nitrogen- or sulphur-containing xenobiotic(s) present in ordinary foodstuffs of plant origin yet absent from synthetic preparations are responsible for the induction of hepatic FMO, and Ziegler has suggested that FMO activity is already maximally induced in animals fed on commercial rat chow (24). It comes to mind what, if any, implications these findings may have if extrapolated to humans. Calorie restriction, adequate ascorbate intake and a diet rich in plant material are all correlated with a reduced risk of numerous so-called ‘western diseases’ in man, including cancers, cardiovascular disease, diabetes and other diseases associated with ageing. Only further research will establish whether diets common in the industrialized nations render populations prone to a reduced level of FMO activity and whether this has implications in health and disease. (150).

Sex related differences in the concentration or enzymatic activity of FMO have been observed in rats, mice and rabbits (102). Gender related differences for mouse liver FMO appear to be due to testosterone repression of the hepatic enzyme (151, 152). Rat liver FMO levels, on the other hand, appear positively regulated by testosterone and to be repressed by oestradiol (153). Oestradiol also seems to play a role in determining the relative contribution of FMO in the S-oxygenation of methoxyphenyl 1,3-dithiolane observed in rat liver microsomes (143). Testosterone treatment of female CF-1 mice reduces FMO activity in the liver but not in the kidney (151). Administration of dexamethasone to Sprague-Dawley rats results in a 98% drop in hepatic FMO levels in males whereas in females hepatic levels remain unchanged (154). Devereux and Fouts were the first to document higher FMO activity in rabbit lung microsomes during pregnancy (through measurement of N',N'-dimethylaniline N-oxidation) and the responsiveness of FMO activity in rabbit lung to administration of glucocorticoids (155). Although changes in the hormonal milieu during late gestation appear responsible for induction of FMO in rabbit lung, these same changes appear not to affect hepatic FMO levels in the rabbit, nor pulmonary or hepatic FMO levels in the mouse (78, 156). The levels of FMO in pregnant rabbit lung have since been shown in one study to be elevated at least five-fold to 2-5nmol/mg of microsomal protein, or 12-30% of the total protein, exceeding the amounts of cytochrome P450 by approximately 5- to 10-fold and making them the predominant microsomal protein in this tissue.
Another study (156) reported that whilst FMO activity and amounts increased significantly in rabbit lung microsomes during the latter stages of pregnancy, at the same time these parameters remained unchanged in liver microsomes. Interestingly, the mineralocorticoid deoxycorticosterone mimics the affect of pregnancy on FMO activity in rabbit lung and administration of progesterone or dexamethasone (but not oestradiol or aldosterone) induces FMO2 protein in rabbit lung (145, 156). FMO2 mRNA and FMO2 protein levels were reported to peak in the lungs of pregnant rabbits at days 15 and 28-31, correlating with elevations in progesterone and corticosterone plasma concentrations (38). Subsequent work, however, suggests that whilst FMO2 in rabbit lung can be induced by either progesterone or glucocorticoids, enhanced FMO2 expression during pregnancy is more closely related to progesterone levels (145). This contrasts with rabbit kidney, in which FMO2 expression appears to be regulated by glucocorticoid levels rather than progesterone: FMO2 protein levels in rabbit kidney peak at the same time as plasma cortisol is at its highest level during pregnancy (parturition) (145, 158). FMO2 levels in the bladder, while lower than in lung and kidney, were increased by about two-fold during the 20-28th days of gestation (145). The levels of FMO1 also appear increased during mid- and late-gestation in rabbit liver, probably also because of changes in the concentrations of progesterone and/or glucocorticoids, as administration of these steroids to male rabbits enhances FMO1 mRNA levels four-fold (oestradiol and aldosterone had no effect, indeed, oestradiol pretreatment partially blocked the inducing effects of progesterone) (145). Similar experiments with CD-1 mice have yielded contrasting data, with pregnancy resulting in no detectable alterations in FMO activity in lung but an increase in the placenta (159). In the case of sheep (which have been shown to have distinct hepatic and pulmonary FMO forms (160)), FMO may be induced in the liver but repressed in the lung of pregnant animals (160). In 1970, Heinze et al. (7) described a dramatic (twenty-fold) increase of N,N'-dimethylaniline N-oxygenation in microsomes isolated from the corpora lutea of pigs in the later stages of their oestrus cycle. Furthermore, a five-fold induction in FMO activity has been demonstrated to occur in the placenta of pregnant mice (159). Gonadectomy experiments on young animals have further established the endocrinological influence that exists on regulation of FMO activity (151, 153). Diabetes can apparently induce FMO in mice (increase in imipramine N-oxidation) (161). Other researchers have demonstrated a diurnal regulation of female mouse liver FMO activity thought to be mediated by cortisol levels (124). Hypophysectomy of male rats has been shown to lead to a reduction in liver FMO activity (150). Administration of growth hormone or testosterone to these hypophysectomized rats only partially restored hepatic
FMO activities. In addition, the hypophysectomized male rats had enhanced FMO activity in the kidney but lowered activity in lung. Castration also reduced hepatic FMO activity in the male rat, though not to the same extent as hypophysectomy (150). In contrast, the hypophysectomized female rat showed no decrease in hepatic FMO activity: rather, there was evidence for an increase in activity (150). However, as with the male rats, hypophysectomy lowered lung FMO activity in females.

These findings have several implications. The plasticity of FMO expression suggests that the secondary side effects of certain drugs could be dependent in some cases on the extent of FMO expression in a given individual and the concomitant effects on xenobiotic metabolism that this would bring about (such interactions are well documented in the case of the cytochromes P450). Furthermore, it gives a rough indication of some of the upstream cis-acting regulatory elements that could be expected to be found within FMO genes once appropriate sequence data have become available.

1.5 Ontogenic and tissue-specific expression of FMOs

Some mention has already been made (Sections 1.3, 1.4 and 1.5: The family of mammalian FMOs, FMO activity in relation to endocrine and nutritional status and Ontogenic and tissue-specific expression of FMOs, respectively) regarding the observation that each individual FMO form appears to have a characteristic pattern of expression according to the tissue examined and stage of development of the organism. Indeed, this is the reason for the finding that different tissues have characteristic but distinct FMO catalytic abilities (162, 163). Furthermore, it is apparent that such patterns of expression vary between mammalian species and within species according to sex and endocrinological status such as pregnancy and the menstrual cycle. However, between human individuals, there seems little evidence for significant inter-individual variation in the pattern of expression of the FMO genes, to the extent that is witnessed in the case of the cytochromes P450, for example (164, 165).

Perhaps the best characterized mammal with regards to the pattern of tissue-specific expression is the rabbit, although patterns have been observed in other species (75, 85, 102, 127, 140, 166, 167). I have already cited (Section 1.3: The family of mammalian FMOs) that, as long ago as 1984, Williams et al. demonstrated that the predominant FMO form in rabbit lung (now known as FMO2) was immunochemically and catalytically distinct from the predominant
form of rabbit liver (now known as FMO1) (74). Soon after, it was shown by a
different group that rabbit kidney possessed FMO catalytic activity,
immunoreactivity and substrate specificity indicative of the presence of both
FMO1 and FMO2 (103, 127) (and now we know that rabbit kidney expresses
all five known FMO forms (38)). Subsequently, it has been shown that FMO2 is
also expressed in rabbit respiratory tract (168) and the site of FMO2 expression
within rabbit lung is predominantly localized within nonciliated bronchiolar
epithelial (Clara) cells (169). Rabbit FMO3 mRNA has been detected in liver but
not in lung (100). FMO4 mRNA was found in another study to be present in
rabbit kidney but not rabbit lung or rabbit liver (85). FMO5 mRNA has been
found in highest amounts in the liver of the rabbit, in lesser amounts in the
rabbit kidney but not at all in rabbit lung (140).

Shehin-Johnson et al. have published a comprehensive study of the tissue-
specific expression of FMO1 and FMO2 in the rabbit. Measurement was made
of FMO1 and FMO2 mRNA amounts (via northern hybridization analysis with
cDNA probes) and protein amounts (via Western blot analysis) in male and
female liver, lung, kidney, oesophagus, intestine, nasal mucosa (maxilloturbinates
and ethmoturbinates) and gonadal tissue (170). Both forms of measurement
(mRNA and protein) were in broad agreement with each other, revealing that
FMO1 expression was highest in liver and intestine, followed by
ethmoturbinates, maxilloturbinates and least of all, female kidney. FMO2
expression, on the other hand, was highest in lung, followed by
maxilloturbinates, ethmoturbinates, oesophagus and kidney. FMO2 expression
was found to vary more between the sexes than FMO1 expression, with females
having higher amounts of FMO2 mRNA in oesophagus, nasal mucosa and
kidney. These apparent differences in FMO1 and FMO2 amounts between
different tissues were confirmed through measurement of relative levels of
catalytic activity. N-oxidation of N',N'-dimethylaniline (DMA) was measured as
a marker of FMO catalytic activity in general, whereas the oxidation of several
thiocarbamides capable of differentiating between different FMO isoforms gave
more specific information. Highest levels of DMA N-oxide formation were
detected in liver, lung and nasal mucosa and, apart from intestine (in which
amounts of protein and levels of catalytic activity were less than would have
been predicted from mRNA amounts), levels of DMA N-oxidation were
proportional to the mRNA and protein amounts previously measured. The
pattern of thiocarbamide S-oxidation was also found to reflect the findings of
the northern hybridization and western blotting studies with respect to FMO1
and FMO2 expression. The finding in this study that, of the tissues examined,
only the nasal mucosa expressed both FMO1 and FMO2 to any significant
degree contrasts with an earlier study by Sabourin et al. (168) which found only FMO2 in this tissue. Nevertheless, it will be interesting to find out what role FMO(s) play in olfaction: removal of noxious odorants, detoxification of xenobiotics and perhaps even odorant processing seems likely.

There is now a fair amount of data available regarding the pattern of FMO tissue-specific expression in humans. Sadeque et al. (163) examined the stereoselective formation of p-tolyl methyl sulfoxide from the corresponding sulphide in detergent-solubilized microsomes from human adult liver, adult kidney, and foetal liver, in order to compare the functional activities of the resident FMO(s). Solubilization with detergent was performed to eradicate the contribution that cytochrome P-450 would make to the net stereochemistry. Consistent with studies in experimental animal livers, solubilized microsomes from human foetal liver and adult kidney formed (R)-p-tolyl methyl sulfoxide in greater than 86% enantiomeric excess. Sensitivity to methimazole inhibition and marked thermolability in the absence of NADPH strongly suggested that these reactions were being catalyzed by FMO. However, solubilized adult human liver microsomes displayed little stereoselectivity (0-40% enantiomeric excess) for the formation of (R)-p-tolyl methyl sulfoxide, despite also displaying several of the characteristics of an FMO-dependent process, including sensitivity to methimazole inhibition and NADPH protection against heat inactivation. Human tissue metabolite profiling was further studied by using the ethyl, propyl and isopropyl p-tolyl sulphides. Parallel changes in product stereochemistry as a function of increasing steric bulk were observed with the foetal liver and adult kidney tissue, whereas an anomalous profile was again observed with adult human liver. These data are consistent with the presence of functionally discrete complements of FMO in detergent-solubilized adult and foetal human liver microsomes. More precise detail regarding which FMO forms are responsible for these catalytic differences has been provided by Dolphin et al. and Phillips et al. (84, 86, 104, 133). I have already noted how Dolphin et al. successfully used northern hybridization analysis to detect the presence of FMO1 mRNA in total RNA from human foetal liver and adult human kidney, but failed to do so in the case of adult human liver (Section 1.3: The family of mammalian FMOs, Subsection 1.3ii: FMO1) (86, 104), and how this clearly contrasts with other mammals such as pig and rabbit, in which FMO1 constitutes the major FMO form in the adult animal (99, 100). The same form of analysis fails to detect, or detects only very small amounts of FMO4 mRNA in total RNA isolated from human adult liver, kidney and lung, or foetal liver (84).
A more specific, sensitive and quantitative measure of mRNA amounts within a given tissue can be undertaken with the RNase protection technique. This has confirmed the presence of FMO1 mRNA within human foetal liver and human adult kidney and shown that it is also present within human foetal kidney and, in much lower amounts, in human foetal lung and human adult skin (86). It was also detected within one of three human foetal brains examined (86). Once again however, FMO1 mRNA was found to be absent from human adult liver (86). Turning to FMO3, RNase protection revealed the mRNA to be present in low abundance within human foetal liver and lung, and adult kidney and lung, but in much greater amounts in human adult liver (86). FMO4 mRNA, on the other hand, is shown to be present in relatively small amounts in numerous foetal and adult tissues, and so the FMO4 gene appears to be expressed in a constitutive fashion (86). Although humans, in common with other mammals, were found to express FMO2 predominantly in the adult lung at relatively low levels, we now know that in most ethnic groups translation of the mRNA does not produce a functional FMO2 enzyme (as I have discussed earlier). Perhaps then, the original term 'lung form' for FMO2 holds true across most mammals, even if this form is sometimes also expressed in some other tissues such as kidney. Finally, although few data are available on the expression of FMO5, in adult humans it would appear that it is expressed in the liver, but in lower amounts than FMO3 (86).

1.6 Genome localization of human FMO genes

All five known human FMO genes have been localized to the long arm of chromosome 1 (1q) (84, 104, 171, 172). In each case, this was achieved by means of screening panels of human-rodent somatic cell hybrids by the polymerase chain reaction (PCR) technique. It is worth briefly considering the nature of somatic cell hybrids, given their importance to the experimental work described later in this thesis. Somatic cells in culture will only fuse infrequently to form a binucleate heterokaryon. It was discovered, however, that certain viruses with similar lipoprotein envelopes to the plasma membranes of human cells, such as the Sendai virus, have properties that can accelerate this process. Unlike most viruses, the Sendai virus makes numerous points of contact with the membrane of its host cell via a glycoprotein in the viral membrane. If two cells are positioned close to each other then the virus can simultaneously attach to both of them. As the virus is so small in comparison to both cells, they are held very close to each other. In many cases this will result in the membranes of the two cells fusing, producing a single binucleate heterokaryon. If, for example, a
suspension of human fibroblasts are mixed with mouse tumour cells in the presence of Sendai virus (inactivated by ultraviolet light), then the virus mediates fusion of both cell types. Incidentally, it has been found that the addition of polyethylene glycol, which causes cell plasma membranes to adhere to those of adjacent cells, produces the same effect without the need for a virus. Following cell fusion, both nuclei eventually fuse to form a uninucleate cell line. Through a process that is poorly understood, as the cells divide, human chromosomes are gradually eliminated in a random manner. A way of arresting this process, before all human chromosomes are lost, is to use mouse cells genetically deficient in some function (often a nutritional one) so that, if continued growth is to occur, the function must be supplied by the human genome. Such a selective technique allows the maintenance of hybrid cells that have a complete set of mouse chromosomes and a small number, or even just one, human chromosome(s). Sometimes, a human chromosome may not be complete and consist only of the long or short arm. The development of stains such as quinacrine and Giemsa, that generate a highly specific and constant pattern of banding for each chromosome at metaphase, allows rapid identification of the remaining human chromosome(s). Once the karyotype has been established, then the cell line can be maintained. Eventually, a battery of cell lines, each with a different complement of human chromosome(s), become available for analysis. If the presence of a human gene of interest is screened for from a panel of these cell lines, either through seeking to identify the presence of the gene product (for example, by testing a cell line biochemically for a particular enzyme or immunologically with a specific antibody preparation), or the gene itself (via hybridization techniques or PCR), then by a process of logical deduction the human chromosome (or chromosome arm) on which the gene resides can be determined. Human-rodent (rat, mice and hamster) somatic cell hybrids have been extensively used in this manner for somatic-cell genetic research. The development of somatic cell hybrids has revolutionized the mapping of human chromosomes (173-177).  Prior to their development, geneticists had to rely on the study of family pedigrees in order to deduce linkage of various traits.

Shephard et al. refined the localization of human $FMOI$ further to 1q23-25 by in situ hybridization of human $FMOI$ cDNA to human metaphase chromosome spreads. In this thesis, I will show how yeast artificial chromosomes, bearing human genomic DNA inserts that were known to contain human $FMO$ genes, were used to further localize these genes by fluorescence in-situ hybridization (FISH) analysis of human metaphase chromosomes. The results obtained show that human $FMO1, FMO2, FMO3$ and $FMO4$ are clustered together at 1q23-24,
whereas *FMO5* is further removed towards the centromere at 1q21. It is intriguing to speculate on the possible significance of these findings, such as whether the clustering of *FMO1*, *FMO2*, *FMO3* and *FMO4* has any bearing on the regulation of their expression, or if it is mere coincidence that *FMO5* is both genomically separated from other members of the *FMO* gene family and unique in its apparent status as a non-drug metabolizing enzyme (140, 141). Certainly, it would seem unlikely that these genes would remain so tightly linked since their divergence from the common ancestral gene were it not for the fact it was an intrinsic requirement for their normal function. If one looks at the cytochromes P450 gene family, for example, members of this family that apparently diverged more recently than the *FMOs* are located on different chromosomes (178-180). It is tempting to draw comparisons with other more characterized gene families, such as the β-like globin gene cluster: in this instance, five functional genes (in addition to two non-functional pseudogenes) are located within a region of about 50kb on human chromosome 11 and members of this family are expressed in a tissue- and developmental-specific manner (181). Also, from the standpoint of understanding the evolution of the *FMO* gene family, it would be interesting to elucidate the genomic locations of *FMO* genes in other mammalian organisms. To date, there appears to have been no reports of this, however.

1.7 *FMO* gene regulation

So far, examples whereby the administration of a defined exogenous agent leads to an alteration in levels of FMO or FMO activity in a given tissue remain rare (one of the best examples in this regard is indole-3-carbinol, a component of Brassica vegetables, and more will be discussed on this later). This is by way of contrast to the cytochromes P450, for which there is abundant evidence for the influence of xenobiotics, both substrate and non-substrate, on levels of activity and gene expression (11). Rather, as I have already discussed (Sections 1.4 and 1.5: *FMO activity in relation to endocrine status* and *Ontogenic and tissue-specific expression of FMOs*, respectively) levels of FMO activity appear more responsive to endogenous parameters (in particular, endocrine status) and tissue specific factors acting at the level of transcription. Examples I have given include how FMO activity has been observed to increase as a function of gestation (109, 110), age (145) and glucocorticoids (109, 110) and to be under the control of sex steroids (150, 151). Furthermore, these studies and others demonstrate that variations in levels of FMO activity between different tissues, sex and stages of development differ according to the form being examined.
To understand how this is achieved at the molecular level clearly requires analysis of genomic regions flanking each of the FMO genes. So far, very little has been achieved in this regard. In Chapter 4 of this thesis (Isolation of YACs bearing FMO-containing human genomic inserts), I describe how human genomic fragments, as inserts of yeast artificial chromosomes, were isolated bearing each of the five known FMO genes. Further characterization of these clones holds the potential for making advances in understanding the molecular mechanism regulating human FMO gene expression. During the earlier discussion of FMO3, I cited recent work by Dolphin et al. that reported how analysis of approximately 400bp of immediate 5' flanking sequence of FMO3 (134). identified three TATA box-like sequences, an AT-rich sequence (TAATAT) and two AATAAA hexamers, located 24bp, 77bp and 270bp, respectively, upstream of the approximate transcriptional start site of the gene.

Some limited data on the 5' flanking sequences of the rabbit FMO1 and FMO2 genes have been published by Wyatt et al. (108). One glucocorticoid response element was located in the 5' flanking region of the FMO1 gene and three in the 5' flanking region of the FMO2 gene. Furthermore, in the case of the FMO1 gene, numerous TATA and CAAT box consensus sequences were identified. Regarding the FMO2 gene, a single putative TATA box was identified in addition the so-called enhancer core sequence (GTGGAAA).

Further data on the flanking regions of the rabbit FMO2 gene have been published by Shehin-Johnson et al. (182). This was also achieved by the analysis of genomic clones isolated from a rabbit genomic DNA library (constructed with lambda EMBL3 vector) after probing with rabbit FMO2 cDNA. The use of primer extension analysis provided a putative transcription start site that extended the previously published cDNA sequence by 17bp. Sequencing and computerized analysis of the isolated rabbit FMO2 flanking sequences depicted a region non-typical of classical TATA promoters (183) but with considerable similarity to initiator (Inr) promoter sequences (184). The significance of this is as follows. Transcription initiation by mammalian RNA polymerase II is achieved through the interplay of basal cis- and trans-acting factors, the most celebrated of which is the cis-acting ‘TATA box’ and its cognate trans-acting factor, TFIID (185), which promotes the assembly of other factors into a preinitiation complex (186). Not all genes, however, have been found to contain obvious TATA boxes, and the rabbit FMO2 gene appears to be one of them. Those that do not have a TATA box have been classed into two categories. The first category consists of genes containing the guanine/cytosine-rich HTF islands (DNA domains rich in cleavable sites for 5-methyl-cytidine/guanosine-sensitive restriction enzymes) (187), found primarily
in housekeeping genes. These genes usually contain several transcription initiation sites spread over a fairly large region. HTF islands were absent from the FM02 upstream sequences examined in this study. The second category of non-TATA box gene promoters, of which the FM02 gene promoter appears to be one, includes those that are not guanine/cytosine rich. Unlike the housekeeping genes, genes within this category are not necessarily constitutively active and transcription is initiated at only one or a few clustered sites. Promoters of this kind are termed Inr and, unlike the TATA box, which is approximately 30bp upstream of the transcription start site, the Inr element contains within itself the transcription start site (184). TFIID is also an integral component of the transcription machinery for the TATA-less promoters, including those using the Inr element. However, unlike TATA promoters, binding of TFIID directly to the Inr element does not provide the nucleation site for transcription complex formation. Rather, recognition by an initiator sequence binding protein (ITF) provides a means by which an ITF-dependent transcription-competent complex can assemble (188). Inr elements share a common CA or CAAC core of nucleotides, as typified by the promoters of numerous genes such as the human leukocyte interferon gene (LeIF-J) (189). Analysis of the FM02 flanking promoter sequence also revealed the existence of several other recognition sequences for known transcription factors, including thyroid transcription factor 1 (TTF-1), polyomavirus enhancer activator 3 (PEA3) and Sp1. The PEA3 DNA binding element (190, 191), a member of the E26 transformation specific (Ets) transcription factor gene family (192), was located in three positions: -40, within the first intron at position +127 and within exon 2 at position +431. It is conceivable that transcription factors such as PEA3 could interact with TFIID and ITF for transcription initiation. A PEA1/API binding element was also located at position +334. Interestingly, cooperative binding between PEA1 and PEA3 has been reported to activate transcription (191, 193), and an Ets family member closely resembling PEA3 in DNA binding specificity interacts with the Inr element in the mouse deoxynucleotidyl transferase gene (Tdt) and is necessary for transcription initiation (194). Ets proteins known to bind the PEA3 motif found in the rabbit FM02 promoter include PEA3, Ets-1, Ets-2, Erg, Elk-1 and PU.1/Spi-1 (190, 193). The presence of the recognition site for TTF-1 is also probably significant. TTF-1 has been shown to enhance lung epithelial cell-specific expression of the murine surfactant protein A (SP-A) and Clara cell 10-kDa protein (CC10) genes (195, 196), and TTF-1 has been linked to the expression of all pulmonary epithelial genes analyzed to date (195). The reader may recall that the primary site of FM02 expression in rabbit lung was shown (by immunohistochemical analysis) to be within the Clara/typeII cell population (169). As methylation has

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been shown to be an important factor in the regulation of expression of numerous genes (197), Shehin-Johnson et al. (170) set out to determine whether it also plays a role in the regulation of rabbit FMO2 gene expression. The DNA region spanning -1100 to +430 of the rabbit FMO2 gene was used as a probe for methylation differences in genomic DNA between that derived from a highly enriched Clara/typeII cell population and that derived from hepatocytes (in which tissue there is no evidence of FMO2 expression in the rabbit liver). Digestion of this DNA region with methylation-sensitive restriction enzymes revealed no differences in the cutting patterns between the genomic DNA extracted from the Clara/typeII cells and the genomic DNA extracted from hepatocytes. It can therefore be concluded that the methylation status of the immediate flanking region of the rabbit FMO2 gene plays no part in the tissue-specific expression of this gene. However, the same region of DNA (-1800 to +430) was tested for the differential presence of DNase I-hypersensitive (DHSS) sites and/or domains, because such sites have been associated with the activation of gene transcription (198, 199). Interestingly, at least one DHSS site was found to be specific for the genomic DNA isolated from the Clara/typeII cells and this region mapped to where consensus sequences for PEA3 (-40) and TTF-1 (-29) elements were previously located.

1.8 Heterologous expression of mammalian FMOs

Given that the five known members of the FMO gene family have been maintained through mammalian evolutionary history and are expressed differentially according to tissue and stage of development, each must have a distinct function in its own right. Defining the separate roles of the FMOs necessitates studying their respective substrate specificities and enzymatic properties in isolation. Studies with microsomal preparations can be complicated by the likelihood that any given tissue can have detectable amounts of more than one FMO form, even if one form is clearly predominant over others. Heterologous expression of the cDNAs of the respective FMOs provides a solution to this problem, provided that the expression system used generates an enzyme with identical properties to that which is found in the natural state. The first FMO cDNA to be expressed in E.coli was pig FMO1 (46). N-oxygenation of tertiary amines was compared between highly purified preparations of this enzyme and highly purified enzyme from pig microsomes. Kinetic parameters were similar between the two preparations when typical FMO substrates (for example, chlorpromazine and trifluoperazine) were used (46). However, when stereoselectivity of sulphide S-oxygenation was compared, differences were
noted. This has been attributed to the microsomal FMO enzyme being N-glycosylated, a form of post-translational modification not carried out by prokaryotes. Alternatively, it has been suggested that the presence of detergent was responsible for this effect (48, 97). When similar comparisons were made between human FMO3 expressed from the cDNA in *E.coli* and human liver microsomes (known to express FMO3 as the major FMO form), however, little in the way of difference was observed, including in N- and S-oxygenation stereoselectivity (46, 47, 122, 200, 201). Overall, it is accepted that although it is possible N-glycosylation may play a structural or functional role in the case of pig FMO1 and perhaps some other FMO enzymes, as a general rule post-translational modification is not a requirement for normal FMO activity. Hence, it would seem that expression in *E. coli* is an adequate system for studying individual FMO enzymes.

The cDNAs of rabbit FMO2, FMO3 and FMO5 have been expressed in active form in *E.coli* and their activity towards a number of traditional FMO substrates examined (89). In the case of FMO5, the enzyme failed to act upon a number of so-called typical FMO substrates, such as methimazole, chlorpromazine, prochlorperazine, imipramine, N',N'-dimethylaniline, cysteamine and trimethylamine (140). However, n-nonylamine and n-octylamine were N-oxygenated by this enzyme.

Many FMOs have been successfully expressed using expression systems other than *E.coli*. For example, rabbit FMO2 cDNA has been expressed in *Sacchoromyces cerevisiae* and compared with the enzyme expressed in *E.coli* (89). Rat FMO1 cDNA has also been expressed in yeast (166) and rabbit FMO1 and FMO2 cDNAs have been expressed in COS-1 cells (128).

Attempts to express active FMO4 from the rabbit cDNA in *E.coli*, yeast, or COS-1 cells (85), or from the human cDNA in *E.coli* or insect cells (*Spodoptera frugiperda* cells infected with baculovirus) (86) have proved futile so far. It has been tested whether this failure of expression is due to the coding regions of FMO4 transcripts being 60-75 nucleotides longer than the coding regions of the other isoforms, due to a shift in the stop codon to the 3'-end of the consensus position (84, 85). It was found that relocating the stop codon of human FMO4, by a single base change in its sequence, to the consensus position (shifting the stop codon 81 bases in the 5' direction from its normal position) allowed an active form of FMO4 to be expressed in *E.coli* (202). The enzymological properties of this truncated peptide were found characteristic of FMOs.
Further expression of human FMOs in heterologous cells has been reported by Phillips et al. (86). Human FMO1 and FMO3 were each successfully expressed within both *E. coli* and insect cells. Although a comparison between the expression systems indicated that the eukaryotic system is more efficient for producing total amounts of enzyme activity, actual substrate specificity and stereoselectivity were indistinguishable between both systems. This confirms the findings discussed above for other mammalian FMOs that have been expressed in both prokaryotic and eukaryotic systems, with regards to both forms of expression system producing catalytically identical enzymes when compared.

1.9 Role of FMOs in xenobiotic metabolism

Gradually, as awareness and research into the FMOs has increased, the previous assumption that the cytochromes P450 are by far and away the main participants in xenobiotic oxidation has been overturned. Now there can be little doubt that the FMOs are a key component in the monoxygenase system that converts nucleophilic amines, sulphides and other heteroatom-containing drugs and chemicals into more polar, readily excretable metabolites (357). Given that the different FMO forms are present at varying amounts according to the tissue type, stage of development, sex, endocrine status, nutritional status and so forth, increasing our understanding of the role played by the FMOs in xenobiotic metabolism necessitates consideration of not just the liver as the site of action, but also other organs not as commonly associated with drug and chemical oxidation.

Many studies have demonstrated that adult human liver microsomes are efficient in tertiary amine *N*- and sulphide *S*-oxygenation (46, 47, 119, 162, 163, 200, 201, 203-206). Because human liver FMO activity is thermally labile, in order to maintain the expected relationship between FMO activity and FMO immunoreactivity, tissue samples from which microsomes are to be prepared must be snap-frozen immediately. Having done so, it has been shown that adult human liver FMO activity does not vary according to gender, age, smoking history, previous drug administration history or between individuals from which the tissue was obtained (119, 201). In humans, the major hepatic FMO is FMO3, with FMO5 and FMO4 also present, but at lower levels (much lower in the case of FMO4) (86, 133). *N*,*N*-Dimethylaniline *N*-oxygenase activity has been observed in the presence of human foetal liver (107) and adult human renal microsomes (163). In both cases it is likely that FMO1 is primarily responsible,
although other FMO forms are apparently present in significant amounts in human adult kidney. It has been shown that imipramine N-oxygenation, considered a selective functional probe for FMO1 activity, takes place at significant levels in adult human kidney microsomes but is largely absent from adult human liver microsomes (162). On a similar theme, it has been demonstrated that stereoselective S-oxygenations in the presence of adult human kidney and foetal human liver microsomes are comparable but distinct to that seen in the presence of adult human liver microsomes (163).

(S)-Nicotine N-1' oxygenation can be used as a selective functional probe for FMO activity. In the presence of adult human liver microsomes supplemented with cytosolic aldehyde oxidase, (S)-nicotine is converted to (S)-cotinine and (S)-nicotine N-1'-oxide (122). Of the two N-1'-oxide diastereomers that could form, only trans-(S)-nicotine N-1'-oxide was observed. Other monooxygenases, including cytochrome P450 from rat liver, mouse liver, and rabbit lung, also catalyzed the formation of (S)-nicotine N-1'-oxides, but at lower diastereoselectivities (specifically, trans:-cis-(S)-nicotine N-1'-oxide ratios of average value 82:18 (119)). In the presence of cDNA-expressed human FMO3, as in the case of human adult liver microsomes, only trans-(S)-nicotine N-1'-oxide was observed (119). Highly purified rabbit FMO2 and pig FMO1 formed (S)-nicotine N-1'-oxide with trans:-cis-(S)-nicotine N-1'-oxide ratios of 100:0 and 57:43, respectively (121, 122). Thus, pig FMO1 and cytochromes P450 appear considerably less stereoselective toward (S)-nicotine N-1'-oxygenation than rabbit FMO2 and human FMO3. In an apparent attempt by Park et al. to verify that human FMO3 is solely responsible for forming trans-(S)-nicotine N-1'-oxide in the presence of human adult liver microsomes, (S)-nicotine was administered to healthy male smokers via either free smoking, intravenous infusion or dermal patch administration (119). The stereoselectivity of the (S)-nicotine N-1'-oxide metabolite was then measured in the urine of these subjects, revealing the presence of only the trans-(S)-nicotine N-1'-oxide. The conclusion by the authors of the study is that, (a) in adult human males, (S)-nicotine is N-1'-oxygenated with absolute stereoselectivity to the trans-(S)-nicotine N-1'-oxide and formation of this metabolite is a selective functional marker for FMO3; and, (b) the fact that no cis-(S)-nicotine N-1' oxide was observed suggests that non-hepatic (S)-nicotine N-1'-oxygenation in humans (i.e. kidney, intestine and elsewhere) does not occur and that FMO1 makes an insignificant contribution to (S)-nicotine N-1'-oxygenation in humans.

As has already been suggested (Section 1.1: Introduction), FMOs have almost certainly evolved to cope with foreign compounds internalized through the
consumption of foodstuffs, primarily plant materials. It would seem likely that FMOs complement the cytochromes P450 in this regard through being recalcitrant to inactivation by many chemicals present within plants that otherwise readily inactivate the cytochromes P450 and other haemoproteins, as well as preventing the untoward effects of bioactivation of chemicals by cytochromes P450 (6, 18, 19, 24). In the main, FMOs oxygenate naturally-occurring nitrogen- and sulphur-containing compounds to less biologically toxic materials (207, 208). For example, the pyrrolizidine alkaloid plant toxins senecionine, retrosine and monocrataline are efficiently detoxified to the tertiary amine N-oxide in guinea pigs, an animal model known for its high FMO activity relative to pyrrole-forming cytochrome P450 activity (154, 209, 210). By contrast, in a species more susceptible to the toxicity of the plant alkaloids, such as the rat, the opposite is true: pyrrole-forming cytochrome P450 activity is very high and detoxicating N-oxide-forming enzyme activity is very low (48). As discussed above, N-oxygenation of another plant alkaloid, (S)-nicotine, by FMO probably constitutes a detoxification route in animals (including humans), shunting alkaloid substrate away from the metabolic pathway mediated by cytochromes P-450, which generate the electrophilic (S)-nicotine Δ1′,5′-iminium ion. The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been shown to be an excellent substrate for pig FMO1 (211, 212) and tertiary amine N-oxygenation of MPTP affords a polar metabolite that represents a major route for detoxification (211-214). It seems reasonable to speculate that in species with a low level of FMO activity, the majority of MPTP is metabolized via monoamine oxidase type B (MAO-B) to the neurotoxic metabolites 1-methyl-4-phenylpyridinium ion (MPP+) and N-methyl-4-phenyl-2,3-dihydropyridinium ion (MPDP+). In mice, in vitro and in vivo studies show that FMO represents a detoxification pathway for MPTP (213-215). Interestingly, it has been shown that the drugs deprenyl and pargyline, both of which are known MAO-B inhibitors and have been used in the treatment of Parkinson’s disease, are substrates for FMO1 (216) (more will be discussed of MPTP N-oxygenation by FMOs later).

Animal liver FMO activity (i.e., primarily FMO1) catalyzes the N-oxygenations of a wide variety of tertiary and secondary nitrogen-containing chemicals (217-222). There are only a few examples of FMO catalyzing bioactivation to more reactive N-oxygenated metabolites. One is the N-oxygenation of N-alkylarylamines to N-hydroxylarylamines, (223, 224) which in turn become metabolically activated to reactive esters with the carcinogenic potential of arylamines in animals. Another is the N-oxygenation of 1,1-dialkylhydrazines in a process (15) that could contribute to the toxic property of these substances.
One of the determinants of the overall toxicity of a primary arylamine chemical may be dependent on the tendency of the amine to undergo \(N\)-methylation versus direct \(N\)-oxidation. \(N\)-methylation of a secondary amine to yield the tertiary amine provides a substrate for FMO-mediated tertiary amine \(N\)-oxidation, in turn giving the tertiary amine \(N\)-oxide that would represent the detoxification pathway. There are a few examples whereby FMO may contribute to the formation of electrophilic metabolites through nonenzymatic rearrangement (Cope-type elimination reactions) of enzymatically-generated tertiary amine \(N\)-oxides (219, 221, 225). For instance, verapamil \(N\)-oxide is efficiently formed by FMO from the tertiary amine verapamil but decomposes to a hydroxylamine and 3,4-dimethoxystyrene (218). These metabolites may contribute to the cardiotoxicity observed with the parent drug. Finally, secondary hydroxylamines can be metabolized by FMO to nitrones that can hydrolyze to electrophilic aldehydes (18, 222).

Regarding sulphur-containing xenobiotics, this class of chemical provides further examples of reactive metabolites produced by FMO. For example, some thiols, thioamides, 2-mercaptoimidazoles, thiocarbamates and thiocarbamides are efficiently \(S\)-oxygenated by FMO to electrophilic reactive intermediates. Without exception, however, the reactive metabolites do not inactivate FMO but in some cases are sufficiently stable and electrophilic to covalently modify other nearby proteins, including cytochromes P450. For example, spironolactone thiol is \(S\)-oxygenated by FMO to a sulphenic acid (42), which combines readily with any glutathione present to form glutathionyl-spironolactone disulphide (42). Ziegler has speculated that reactions of this kind, which catalyze the oxidation of cellular thiols (such as glutathione) and NADPH, may render the cell susceptible to the toxic properties of other chemicals (24). There is direct experimental evidence that administration of such chemicals as thioureas, mercaptoimidazoles and spironolactone to animals results in elevated levels of oxidized glutathione in vivo. The thiocarbamate functionality is widely used in agricultural products, including many of the herbicides and fungicides in commercial use. Thiocarbamate groups are sequentially \(S\)-oxygenated to \(S\)-oxides and sulphones (226-231). \(S\)-Oxygenation adjacent to a ketone functionality significantly increases the potential for generating powerful electrophilic acylating agents (232). Therefore, it is a possibility that thiocarbamate \(S\)-oxides or thiocarbamate sulphones are responsible for the toxic properties of many herbicides and fungicides in use today, including in fish, which have been shown to have FMO activity (233, 234) and are known in some cases to exhibit adverse toxic reactions to thiocarbamates (235).
1.10 FMOs and the brain

Particular attention has been given to the role of FMOs in xenobiotic metabolism in the brain. Interest from various quarters in this topic is intense, due not least to the large number of drugs in existence that target their action to this organ and the possible role of xenobiotics in the etiology of neurodegenerative disorders such as Parkinson's and Alzheimer's disease. Many psychotropic drugs, such as the tricyclic antidepressants chlorpromazine, imipramine, amitriptyline, nortriptyline and trifluoperazine, have been demonstrated excellent substrates for FMO (6), leading to the formation of their respective N-oxides, which are pharmacologically inactive. In comparison with other organs, however, the study of FMO activity in the brain is made comparatively difficult by its sheer complexity: for example, determining amounts of a given FMO within whole brain is more straightforward but far less informative than elucidating amounts of that FMO within the numerous different compartments of the brain known to have specialized functions.

The presence of several drug metabolizing enzymes, especially the cytochromes P450, have been revealed in brain (236-238). As long ago as 1984, Duffel and Gillespie demonstrated the presence in the rat corpus striatum of FMO-like activity (239). Subsequent research has confirmed that FMO is found within brain, but has produced vague and sometimes contradictory predictions as to the form(s) of FMO that predominate. Whereas the work of Duffel and Gillespie suggested that the FMO activity identified in corpus striatum possessed the catalytic properties of rat hepatic microsomal FMO (now known to be predominantly FMO1), Bhamre and Ravindranath reported that FMO activity in rat brain possessed catalytic properties distinct from that regarded typical of rat liver (8). Specifically, FMO activities for thiobenzamide, N',N'-dimethylaniline, or methimazole were found to be higher for rat brain microsomes than rat liver microsomes. Furthermore, the same group found by western immunoblot analysis that rat brain microsomes did not react with antisera to purified pig liver FMO but did react with antisera to rabbit lung FMO (regarded as predominantly FMO2) (9). Also, addition of rabbit lung FMO antisera resulted in 43% inhibition of FMO-mediated imipramine metabolism by rat brain microsomes. Conversely, Itoh et al. (166) demonstrated that rat FMO1 was moderately expressed in brain as well as lung, kidney and heart. Furthermore, unlike the findings of Bhamre and Ravindranath, Kawaji and coworkers failed to witness any rat brain FMO activity towards thiobenzamide, N',N'-dimethylaniline, or methimazole (240). Rather, Kawaji et al. measured brain FMO activity by fluorometrical determination of benzydamine (BZY) N-oxygenation with high
pressure liquid chromatography. In so doing, the $K_m$ value for rat brain microsomes was found similar to that of rat liver microsomes, but the $V_{max}$ value for brain microsomes was one-hundredth that for liver microsomes. Furthermore, the optimum pH for BZY $N$-oxygenation by brain was found to be more acidic (pH8.5) than that for liver FMO (pH9.0), and $n$-octylamine (a positive effector of liver microsomes) did not activate BZY $N$-oxygenation activity by brain microsomes. Moreover, although the effects of thiourea and thermal stability were both comparable between rat brain and liver microsomes, anti-rat liver FMO antisera inhibited levels of BZY $N$-oxygenation by solubilized rat brain microsomes by only 30%. Kawaji et al. have more recently succeeded in partially purifying an FMO from rat brain microsomes via successive chromatographies on columns of DEAE-Sepharose and 2',5'-ADP Sepharose (241). Western blot analysis of the preparation with anti-rat liver FMO revealed a single band of 60kDa. The $K_m$ value of this enzyme for thiourea was four-fold lower, but for cysteamine ten-fold higher, than that of purified rat liver FMO (FMO1). Furthermore, the $K_m$ values of the brain enzyme for 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1,2,3,4-tetrahydroisoquinoline (TIQ) and N-methyl TIQ (NMTIQ) were lower than those for rat liver FMO. The rationale for measuring activity against these three substrates lies in the fact that they are all neurotoxins capable of inducing Parkinsonism. MPTP is activated to its pyridinium ion (MPP$^+$) by monoamine oxidase (MAO) type B and has been shown to be metabolized to the non-toxic metabolite, MPTP N-oxide, by pig and rat FMO1 (48, 242). NMTIQ is produced from TIQ by N-methyltransferase and is subsequently activated to its isoquinolinium ion (NMIQ$^+$), a potent neurotoxin capable of inducing Parkinsonism, by MAO (243-245). Interestingly, 1-MTIQ, an endogenous amine found in rat and human brain that has an apparent protective effect against Parkinsonism, is found in lower amounts in the brains of animal models of the disease and decreases with age in the brain (246, 247), was not $N$-oxygenated by the purified rat brain FMO. Neither the brain nor the liver FMO was active towards $n$-octylamine, a substrate for FMO2 (6) and FMO5 (141).

Regarding data on the localization of FMO activity within the brain, as already cited in this Section, Duffel and Gillespie demonstrated the presence in rat corpus striatum of FMO activity matching that of rat liver microsomal FMO activity (239). Bhamre et al. found by immunohistochemical analysis that FMO was localized in the hippocampus, brain stem and other areas of the rat brain (9). Kawaji et al. found FMO activity to be between three and four times greater in the microsomes of the rat olfactory bulbs than in microsomes from rat cortex, cerebellum, midbrain and medulla oblongata (240).
There is some evidence available regarding FMO activity in human brain. Bhamre and coworkers obtained brain tissue from seven road accident victims (248) and demonstrated that purified microsomes from all regions of the brain examined catalyzed the S- and N-oxidation of the classical substrates methimazole and N',N'-dimethylaniline, respectively, as well as the psychoactive drugs chlorpromazine, imipramine and fluoxetine. Western immunoblot analysis revealed immunological cross-reactivity between these human brain microsomes and purified rabbit pulmonary FMO antisera. Such cross-reactivity was not observed in the case of pig hepatic FMO antisera, however. (These data, on the face of it, seem rather confusing as the immunological results suggest the enzyme is probably FMO2 but definitely not FMO1, whereas the substrate specificity is clearly not indicative of FMO2 (76, 77) but does match that of FMO1 and perhaps FMO3 (78, 140). Furthermore, we now know that most humans do not express an active FMO2 enzyme (80)). In addition, immunocytochemical localization using the rabbit pulmonary FMO antisera revealed that FMO abundance was greatest in the neuronal cell bodies of the magnocellular reticular nuclei, colliculi and substantia nigra. Localization of human brain FMO activity to the neuronal cell bodies would indicate that both FMO and cytochromes P450 are colocalized, as the latter have been found in the neuronal cell stroma (249). The same group has more recently published further work on FMO activity within human brain microsomes (250). Once again, the immunological data presented might indicate that FMO2 is the primary FMO form present, as enzyme activity was sometimes inhibited by rabbit pulmonary FMO antisera (93% inhibition of imipramine N-oxidation when measuring metabolite formation by HPLC, but no inhibition of metabolism of methimazole, N',N'-dimethylaniline, fluoxetine or chlorpromazine), and FMO purified to apparent homogeneity from the microsomes cross-reacted with the same antibody. Curiously, although the FMO2 antisera inhibited imipramine metabolism but not that of methimazole, the addition of methimazole was found to inhibit formation of imipramine N-oxide, suggesting that the conflicting immunological data were apparently not due to the presence of more than one FMO enzyme. It is worth noting that as there are no reports of the rabbit pulmonary FMO antisera used by this group (provided by D.E. Williams) having immunoinhibitory properties towards FMO activity, the precise implication of the inhibition of imipramine N-oxidation is open to question. Nevertheless, once more, imipramine N-oxidation was efficiently catalyzed and this is not characteristic of FMO2, although thermolability experiments performed (activity only lost after incubation of microsomes at 45°C for 20 minutes) gave results more indicative of FMO2 than FMO1 (18, 78). The pH optima for FMO activity (8.5), on the other hand, seems more characteristic of FMO1 and other FMOs.
than it does FMO2 (18, 75, 78, 127). Also perplexing was the finding that the FMO purified to apparent homogeneity was of molecular mass 71kDa: the molecular masses of all five known human FMOs have been found in the range 60-63kDa (86). Intriguing though these data may be, Bhagwat et al. have published results from similar characterization experiments performed on FMO activity from rat brain microsomes that parallel some of these findings (251). Once more, FMO activity from brain microsomes catalyses the N-oxidation of the non-FMO2 substrate, imipramine. Again, however, incubation with antibody to rabbit pulmonary FMO resulted in inhibition of this reaction (70.6% inhibition). In contrast to their data with the human brain microsomes, though, the same antisera inhibited methimazole metabolism by 86%. As with the human brain microsomal FMO activity, optimum pH was 8.5, which contrasts with the optimal pH for FMO2 activity (pH10), and thermal lability resembled FMO2 more than FMO1 (incubation of microsomes for 10 minutes at 45°C resulted in 68% loss of activity whereas FMO1 activity is abolished after 5 minutes at this temperature). On this occasion, after chromatographic purification of FMO activity, two distinct proteins were evident that both cross-reacted with the rabbit pulmonary FMO antiserum. The molecular masses of these two proteins, 57kDa and 61kDa, were more in line with that expected of known FMOs, particularly in the case of the latter mass (the authors concede that the lower mass band may have been the result of proteolytic degradation). Furthermore, it was found that the FMO activity of the rat brain microsomes was not inhibited by anionic detergents such as sodium cholate: anionic detergents have been shown previously to inhibit the activity of FMO1 but not FMO2 (78). Finally, when the specific activity of the purified rat brain FMO for the S-oxidation of methimazole was measured, it was found considerably higher than that reported for purified rabbit pulmonary FMO and pig hepatic FMO (74, 251).

When we pause to consider all of the evidence cited above, it is clear that much of it depicts a confusing picture as to which FMO forms are actually expressed in mammalian brain and in what relative amounts. One possible explanation for all of these contradictions might be the existence of a sixth, hitherto undiscovered mammalian form. However, a study recently reported by Blake et al. (252), and a review of the data surrounding mammalian brain FMOs in their discussion, offers a less radical explanation. This group used the reverse transcription-polymerase chain reaction (RT-PCR) to probe whole rabbit brain for the existence of mRNA for any of the five known FMO forms (rabbit brain was used as this was the only mammal for which the group had access to the cDNA sequences of all five FMO forms). We must bear in mind that the comparisons made by this group between their results with rabbit brain and
those of other groups with rat and human brain assume that the pattern of FMO expression remains the same across mammalian species. We already know this not to be the case for hepatic FMO expression at least, so some caution is advisable with these comparisons and in the suggested extrapolation of the rabbit brain FMO findings to other mammalian species. Primer pairs for the RT-PCRs were designed, and subsequently shown, to be specific for each of the five FMO forms in rabbit. Only primers for FMO4 produced an amplification product from the rabbit brain, the identity of which was confirmed by nucleotide sequencing. By way of contrast, the same primer sets successfully amplified products specific to all five FMOs in the case of rabbit kidney and all forms except FMO2 in rabbit liver, findings supported by previous studies using northern analysis with cDNA probes (85, 100, 140). The conclusion drawn is that only FMO4 is expressed in rabbit brain, and this at very low levels. This would be in agreement with the findings of Kawaji et al. (240) and Itoh et al. (166), who found little FMO activity in brain relative to liver, although Itoh et al. have also reported the detection of small amounts of FMO1 mRNA in rat brain (166). It would not be in agreement with the findings of Bhamre and colleagues, however, who reported very high levels of FMO activity in rat brain (8, 9). In defence of their own findings, Blake et al. suggest that the validity of the data reported by Bhamre et al. is open to debate due to the reliance of the latter on the presence of Triton N-101, an uncommon requirement for FMO activity, in their incubation mixtures. Furthermore, Blake et al. call into question the significance of the cross-reactivity and immunoinhibition studies performed by Bhamre et al. with rabbit pulmonary FMO antisera. Apparently, western analysis with the same antisera shows a reaction with more than one protein in rat liver and kidney microsomes (167), whereas immunochemical, northern and catalytic studies have revealed no evidence for hepatic expression of FMO2 in rat or other species (78, 100, 103, 127, 140).

Clearly, a great deal more research needs to be performed before the picture becomes clearer regarding the expression of FMOs, and their role, in the mammalian brain.

1.11 Clinical significance of FMO activity

1.11i Fish-odour syndrome/Trimethylaminuria
Trimethylamine (TMA), a dietary component found within numerous foodstuffs, has a historical place in FMO research, as it was the first FMO substrate discovered for which a lack of metabolism by FMO results in a defined clinical
condition. TMA is the highly volatile aliphatic amine responsible for the odour of rotting fish: bacteria reduce the non-volatile and non-odorous trimethylamine N-oxide (TMAO), present in significant amounts in fish, to TMA. In a similar manner, trimethylamine is released in the intestine by the action of bacteria on certain foodstuffs containing the trimethylammonium \([\text{(CH}_3\text{)}_3\text{+N-}]\) function, such as lecithin, choline, carnitine, ergothioneine and betaine (present in egg yolk, liver, kidney, meats, beans and legumes and many other foods). As has already been mentioned in this Section, another potential source of TMA is marine fish, due to their inherent levels of TMAO (up to 0.5% of bodyweight (253, 254)). Freshwater fish, incidentally, are a poorer source of TMAO. Once again, bacteria (naturally resident within the intestine) reduce the TMAO present within consumed fish to TMA, which is then absorbed and converted back to TMAO, a process commonly termed ‘metabolic retroversion’. It is thought that roughly 50% of TMAO consumed in this manner undergoes reduction to TMA, the rest is absorbed unaltered and excreted. TMA has an unusually low odour threshold (0.9ppm) in comparison with other equally volatile amines, suggesting there may have been a selective advantage in possessing the ability to detect the smell of this compound at low concentrations, presumably as it would assist in the avoidance of rotting food (255, 256). At 100-500ppm, the odour of TMA becomes more acrid and resembles ammonia. In humans, concentrations above 20ppm produce a moderate irritation of the respiratory system and eyes; dermal contact with concentrated solutions can cause severe burns. Oral ingestion of TMA at 15mg/kg has resulted in nausea and ichthyohidrosis.

TMA released within the intestinal tract is rapidly and efficiently absorbed. Needless to say, if unaltered, TMA would be excreted from the body via the sweat, breath and urine, resulting in individuals smelling strongly of its highly objectionable odour. The reason this is patent not the case is that the normal (and only observed) metabolic pathway for TMA is its N-oxygenation to TMAO (257-259), a reaction catalyzed by FMO (260). TMA has been demonstrated an excellent FMO substrate (43), and any TMAO generated is excreted mainly through the urine with no odorous consequences (261). Although it is generally believed that FMO is responsible for the bulk of TMA N-oxidation, it is also possible that the cytochromes P450 make a contribution (260). The average daily urinary excretion of TMA and TMAO is about 1-2mg and 50mg respectively for European Caucasians consuming an average diet (262). The amount of TMAO excreted can, however, rise to several hundred milligrams when the diet contains large amounts of marine fish.
There is, however, a well-documented clinical condition known as trimethylaminuria, or more colloquially ‘fish-odour syndrome’, that is characterized by an inability to \( N \)-oxygenate TMA to TMAO. As one would expect, these individuals excrete TMA in their breath, sweat and urine and so smell strongly of an odour resembling rotting fish (43, 44, 262-266). Although the increased amounts of TMA itself does not appear deleterious to the individual, the resultant psycho-social impact can be devastating. For example, a study reported that for 11 subjects (six females) examined with trimethylaminuria, none had any obvious physical or mental abnormality although the subjects showed various psychosocial reactions including low self-esteem, frustration, anxiety, clinical depression, paranoia, suicidal personality, and addiction to cigarettes, alcohol and other drugs (266). This condition, as I shall discuss further, is an inherited metabolic disorder. Its proper title as such is ‘primary trimethylaminuria’ to distinguish it from the other trimethylaminurias (‘secondary trimethylaminurias’) which are non-genetic in origin and are secondary to other factors such as renal or hepatic disease or overload with TMA precursors. In the latter case, for example, it has emerged that treatment administered to patients suffering from Alzheimer’s disease, tardive dyskinesia and Huntington’s chorea, in the form of large doses of the TMA precursors choline and lecithin, has resulted in spontaneous cases of ‘fish-odour syndrome’, presumably because in these individuals the natural TMA \( N \)-oxidizing capacity had been overloaded (267, 268). Trimethylaminuria has also been reported to result from gynecological infections such as bacterial vaginosis (269). Interestingly, there are chemicals found within drugs and common foodstuffs that can act as inhibitors of FMO and these could, in theory at least, result in secondary trimethylaminuria if consumed in sufficient quantity. These include 1-naphthylthiourea and several goitrogenic substances such as thiourea, propylthiouracil, methimazole and the naturally occurring progoitrin (270, 271). Progoitrin, a thioglucoside that undergoes conversion by intestinal bacteria to the potent goitrogen goitrin, is found within Brassica species such as cabbage, cauliflower, brussel sprouts and swede and is a known inhibitor of TMA \( N \)-oxidation (272). It has been implicated in the production of foul, fish-smelling eggs by a breed of chicken with a genetically reduced ability to \( N \)-oxidize TMA, following feeding of these chickens on Brassica rapeseed meal (273). Whether excessive consumption of Brassica vegetables could ever result in secondary trimethylaminuria in humans is not known (274). However, when healthy volunteers consumed 100 times the average daily intake of brussel sprouts and swede, no effect was seen on the apparent TMA \( N \)-oxidation capacity (275). Nevertheless, it may be that in certain susceptible individuals (such as the heterozygote carriers for primary trimethylaminuria which we will
discuss later), large-scale consumption of Brassica vegetables may result in a sporadic fishy odour, in a manner not unlike that described above for the 'fishy egg'-producing chickens. The reader should note that, for convenience sake, 'primary trimethylaminuria' shall be simply referred to as 'trimethylaminuria' from this point onwards in the text.

The study of individuals with trimethylaminuria has provided further, indirect, evidence that the primary catalyst for TMA N-oxidation is FMO. That (S)-nicotine N-1'-oxygenation is a selective functional probe of FMO activity (48, 121) has already been discussed above (Section 1.9: Role of FMOs in xenobiotic metabolism) and this reaction was found to be defective in patients with trimethylaminuria (44). Furthermore, administration of methimazole, a recognized FMO inhibitor (276), reduces the formation of (S)-nicotine N-1'-oxide from nicotine administered as chewing gum preparation to human volunteers (277).

The presence of TMA in human urine was established as early as 1856 by Dessaignes and reports of individuals with a peculiar fish-like odour have been around for some time. Indeed, written accounts of individuals with an intense body odour reminiscent of rotting fish date as far back as 1735 (278), and it appears that Shakespeare was an observer of this condition when he wrote:

"What have we here? a man or a fish?
dead or alive?
A fish, he smells like a fish...a
a kind of not-of-the newest poor-john"
(The Tempest Act 2, Scene 2. Source: R. Ayesh and R.L. Smith)

The first clinical case report of an individual with trimethylaminuria was published by Humbert and coworkers in 1970 (265). It details a six year-old girl with Turner's syndrome, splenomegaly and haematological abnormalities, in addition to the characteristic rotting fish odour of trimethylaminuria. Gas chromatographic analysis of the patient's urine revealed abnormally large amounts of trimethylamine (TMA), a finding confirmed by mass spectrometry. Administration of 15mg/kg TMA/day resulted in the odour worsening in the case of the patient, but not so when repeated on selected healthy individuals.

It was shown by Higgins and coworkers in 1972 that TMA N-oxidation in the liver was catalyzed by a microsomal mixed function oxidase, in other words FMO (279). Furthermore, it was demonstrated that such enzymatic activity was deficient in a liver biopsy taken from the patient reported by Humbert et al.
This would suggest that the liver constitutes the major site of TMA metabolism, a conclusion supported by the finding that a liver biopsy sample taken from a patient with Noonan's syndrome and trimethylaminuria revealed a Michaelis constant ($K_m$) value of TMA for TMA $N$-oxidation several times greater than that observed from the livers of unaffected subjects. More recent data from oral administration studies, showing a high first-pass metabolic effect for TMA, provides further confirmation.

As the first clinical report of an individual with trimethylaminuria detailed a patient also suffering from Turner's syndrome and other abnormalities, it was initially questioned whether trimethylaminuria is invariably associated with other clinical conditions of this kind. However, it soon became apparent that individuals with syndromes very similar to that afflicting the patient described by Humbert et al. do not necessarily also present with trimethylaminuria. Nevertheless, Shelley and Shelley have reported the case of a 31 year-old man institutionalized since early childhood with congenital problems, possibly a variant of Noonan's syndrome, and the symptoms of trimethylaminuria. The individual concerned had a pronounced rotten fish odour and trimethylaminuria was confirmed by gas chromatographic analysis of a urine sample. Dietary restrictions and antibiotic treatment completely eliminated the odour (these are the two forms of treatment commonly used to combat trimethylaminuria and are discussed further below). Furthermore, Chen and Aiello reported the case of an individual with Prader-Willi syndrome, trimethylaminuria and del(15)(q11q13), and speculate that the area of this deletion may include the gene responsible for trimethylaminuria.

The first attempts to offer treatment to sufferers of trimethylaminuria consisted of dietary manipulation to reduce intake of those foodstuffs already mentioned which result in TMA release in the intestine. Although such a strictly controlled diet is perhaps difficult and tedious for the individual concerned due to the number of 'banned' foods involved, it has often been found highly successful in reducing the objectionable body odour. Unfortunately though, the treatment has not been found universally successful. In a study reported by Danks and coworkers, four patients with trimethylaminuria were treated by manipulation of dietary intake, yet only one of these individuals responded positively. However, treatment of the three unresponsive patients with the antibiotic neomycin sulphate, the intention being to reduce levels of intestinal bacteria to such an extent that TMA release was substantially reduced, did result in the fish odour being eliminated from one of them. Thus, antibiotic administration represents another option in the treatment of this condition.
although it must be questioned what side effects may arise from maintaining this treatment long-term. Aside from the implications for treatment of trimethylaminuria, this study also underlines the observation that there appear to be different grades of severity of trimethylaminuria: methods leading to a reduction in the 'dose' of TMA received from the diet work for some patients but not others and there is the anecdotal observation that some patients tend simply to smell worse than others. Presumably, patients unresponsive to the elimination of TMA-releasing foods are nevertheless obtaining enough TMA-producing compounds from other foodstuffs to overload what little TMA oxidizing capacity they possess, a capacity which would seem to be greater in those individuals for whom an identical diet does successfully eliminate the odour. The same principle applies to those who show a differential response to antibiotics.

Trimethylaminuria is apparently the only known disease characterized by a peculiar body odour and not directly associated with developmental abnormalities. However, a number of case studies have revealed the unfortunate psycho-social consequences of trimethylaminuria. One such case was among three reported by Lee and coworkers in 1976, all involving children (284). It concerned a six-year-old boy of normal physical and intellectual development whose mother had first noticed the characteristic fishy odour at 18 months of age. The odour was episodic in its presence and worse in summer. Sadly, the child was apparently suffering victimization at school as a result of his body odour. There was one unaffected sibling and non-consanguineous, unaffected parents. No family history of trimethylaminuria was apparent. After biochemical tests revealed no abnormalities, the boy had fish eliminated from his diet and this did meet with some success, although during hot weather the odour would still sometimes return. The second case reported by Lee et al. was of a two-year-old boy of normal development and intelligence whom the mother had first noticed to possess a fishy odour at 3 months of age (although the odour apparently seemed to be getting worse as the child got older). At this stage the mother was breastfeeding the child and it was noticed that a correlation existed between the consumption of fish and eggs by the mother and the appearance of the odour in her child (presumably because higher levels of TMAO were appearing in her milk at this time). The parents of the child were not consanguineous and no family history of trimethylaminuria was known. However, one of two siblings was also a diagnosed trimethylaminuria sufferer and this individual formed the basis of the third case study by Lee et al. The affected sibling was younger and once again the mother noticed the odour at three months of age.
At the time the study was reported, the child was 15 months old and developing normally.

Another case report which describes well the misery of trimethylaminuria has been published by Todd (285). It describes a thirteen-year-old male who presented with the characteristic fishy odour. Since the age of ten, he had visited doctors eleven times, only to be generally told that bodily odours are a common complaint of young boys which can be remedied by better personal hygiene. As the level of personal hygiene makes no difference to this condition, the boy continued to suffer socially from an almost intolerable fishy odour. Although the boy’s school record revealed that initially his academic performances had been outstanding, these had later dramatically declined to the extent that he had frequently been dismissed from school, often as a result of fights with other children over the constant ridiculing. The parents reported that their son had no friends to speak of and that he frequently played truant from school and appeared overtly depressed. Matters were not helped by the considerable guilt experienced by the boy when the parents failed to sell their house on six occasions as a result of the potential buyers being deterred by the rotten fish smell in the house. There was no history in the boy’s family of trimethylaminuria, including his two siblings, and a professional examination of the boy revealed him to be normal in all other respects, apart from appearing very quiet and somewhat introverted. The fishy odour was noticeable over the whole body but particularly so on the feet and axillae. Analysis of urine samples by gas chromatography revealed the presence of an abnormally large amount of TMA. By reducing the intake of fish and choline-containing foods, levels of urinary TMA were drastically reduced and the objectionable odour was eliminated. Following this successful intervention, the boy’s school performances showed a dramatic improvement to the extent that he began achieving A-grades. Furthermore, he made many new friends, started dating and his parents noted that the apparent depression and related symptoms had disappeared.

Unfortunately, the initial lack of awareness of how to recognize, diagnose and treat trimethylaminuria by the medical community resulted in a catalogue of similarly distressing case histories. Problems have often been made worse by the fact that the odour is frequently episodic, depending for example on what has been eaten in the previous 24 hours. Thus, it may be that the trimethylaminuria sufferer upon consulting a doctor will not have a noticeable odour and that even if they do, the doctor may have a poor sense of smell or efficient air conditioning will dissipate the odour. The doctor may then diagnose the
individual as suffering from some type of psychological abnormality or that he/she is simply overtly self-conscious about their body odour.

Research into a possible genetic basis for trimethylaminuria began with the study of family histories of affected individuals and the testing of siblings and parents for TMA oxidizing capacity. Spellacy and coworkers reported that the father of a patient with trimethylaminuria excreted abnormally large amounts of TMA upon challenge with choline (286). Brewster and Schedewie (287) reported that the oral challenge of choline (50mg/kg) to the mothers of three trimethylaminuria patients resulted in levels of TMA being excreted which were higher than those witnessed for 76 healthy volunteers. Taken together, the studies suggested a genetic basis for trimethylaminuria.

Much of the rest of the work that has been performed on the inheritance of trimethylaminuria has been carried out by Al Waiz and coworkers, who have analyzed the TMA N-oxidative capacity of randomly selected volunteers, trimethylaminuria sufferers and their families (262, 275, 288). From the data they present it can be concluded that: (a) there is a gene responsible for trimethylaminuria that is inherited in an autosomal, recessive manner; (b) carriers are identifiable by their impaired ability to metabolize large quantities of TMA (600mg or more) relative to non-carriers; (c) under normal dietary conditions, both normal individuals and heterozygote carriers may be indistinguishable in their ability to excrete TMAO as a percentage of all TMA and TMAO excreted (values for heterozygotes ranging from 69 to 98% and for normal individuals from 94 to 99%), whereas homozygous sufferers are immediately identifiable by this criteria (values ranging from 10 to 25%); and (d) a study of 169 randomly selected British white people identified two definite heterozygote carriers, although there may have been others that were not detected. Therefore, it has been estimated that the incidence of heterozygote trimethylaminuria carriers in the British general white population probably lies within the range 1-2%.

Much research has been carried out concerning the nature of TMA N-oxidation. For example, it has been shown that 90-99% of an administered dose of TMA is excreted within the first 24 hours as TMAO or TMA (the ratio between the two being at least 20:1 for normal individuals and approximately 2:1 for trimethylaminuria sufferers) (257). Other studies have sought to determine whether TMA undergoes metabolic transformation to compounds other than TMAO, specifically dimethylamine (DMA), which is relatively odourless (olfactory potency approximately 0.01 that of TMA). As long ago as 1965 (289), it was suggested that the ingestion of choline may result in the
excretion of some of the absorbed TMA as DMA, although it was felt that the majority of DMA normally found in the urine was of endogenous origin, perhaps arising through the methylation of methylamine, which is normally derived from sarcosine. More recently, however, Al Waiz and coworkers have shown that when normal individuals are challenged with large doses of TMA (600mg), the quantity of DMA excreted does not rise significantly (290). Furthermore, trimethylaminuria patients were found not to excrete significantly elevated levels of DMA. Thus, it is concluded that the metabolism of TMA does not involve N-demethylation but rather would seem to consist exclusively of N-oxidation.

As has already been stated in this Section, TMA N-oxidation is catalyzed by the action of FMO. From the data regarding the inheritance of trimethylaminuria, it would seem that just one of the FMO forms is responsible for the majority of TMA monooxygenation to TMAO and that this is the gene responsible for this condition. The fact that the FMOs have such broad substrate specificity, however, probably means that all FMO forms, apart from perhaps FMO5, can catalyze this reaction to some degree. This is the likely reason for the small, residual level of TMAO (about 10% of total TMA and TMAO) excreted by even the most chronic of trimethylaminuria sufferers, whom we would expect to completely lack the activity of the FMO enzyme involved (some of this residual TMA N-oxidation could be due, at least in part, to cytochromes P450, however). The enzyme responsible for TMA N-oxygenation was initially attributed to human FMO1 until it was discovered that in adult human liver, very little, if any, FMO1 is expressed (85, 104, 200, 201). Quantitative RNase protection assays have indicated that, of the five forms present in man, only FMO3, FMO4 and FMO5 are expressed in adult liver, with FMO3 mRNA most abundant, followed by FMO5 and least of all, FMO4 (86, 133). As FMO5 is apparently a non-typical member of the FMO family, in that it exhibits little activity towards classical FMO substrates (140, 141), it seems unlikely that it is involved in the N-oxidation of TMA. However, heterologous expression of FMO3 in bacteria (46) has demonstrated that it has a distinct substrate affinity for tertiary amines. Based on this alone, FMO3 would appear the likely candidate for the gene responsible for trimethylaminuria. It is interesting to note that in mammals whose major hepatic FMO form is FMO1, such as rat and guinea pig, N-oxidation of ingested TMA is less extensive and appreciable TMA excretion occurs as a result (291, 292). One can only speculate as to why evolution has given humans FMO3 as the major hepatic FMO enzyme, whereas for other mammals it is FMO1. However, it is believed that man's early ancestors became efficient hunters of fish with the advent of tools and so it is conceivable that the need to
cope with greatly increased amounts of TMA occurred at this time. In connection with this, it has been proposed that the modern western diet is deficient in the omega 3 fatty acids that occur abundantly within fish (in the form of eicosopentaenoic acid and docosahexaenoic acid) as a result of the fact that we no longer match the relatively large-scale fish consumption of our ancestors. As a consequence, there is a believed imbalance towards the omega 6 fatty acids in modern diets, supposedly increasing the incidence of heart disease, some cancers and inflammatory disorders such as rheumatoid arthritis (293-296). Lack of intake of omega 3 fatty acids in mothers has even been suggested to have a negative impact on the development of the brain of the foetus and young child (297, 298). Many doctors now accept, as a result, that people in the more affluent industrialized nations should consume more fish. Altogether, the evidence seems persuasive that for most of our evolutionary history, fish has formed a major constituent of our diet and we might expect, with the negative social impact of the TMA odour, there would be a selective pressure to increase the ability to N-oxygenate the increased TMA dietary load.

The most direct molecular evidence for the cause of trimethylaminuria has come recently from Dolphin et al. and would seem to confirm that FMO3 is indeed the gene responsible for this disorder (299). After establishing the internal organization of the human FMO3 gene, each of its eight coding exons were amplified by PCR, both from a normal individual and from an individual with trimethylaminuria. The amplified exons were sequenced and compared for differences. This identified some sequence differences, the most significant and interesting of which was a single base change, a C to T transition, within exon 4. As a result, a CCC triplet coding for proline-153 in the FMO3 sequence from the normal individual becomes a CTC triplet coding for leucine in the individual with trimethylaminuria (for both alleles). The sequence of exon 4 of the FMO3 gene was then determined for the affected individuals’ parents and two siblings. The parents were found heterozygous for this mutation, as would be expected if the mutation was causative of trimethylaminuria. Of the two siblings examined, one was also trimethylaminuric and the other apparently unaffected. The former was found homozygous for the CTC mutation, whereas the latter was found heterozygous for the mutation. Four unrelated, non-trimethylaminuric individuals when examined were all found to be homozygous for the CCC triplet. Thus, it would seem a distinct possibility that in the case of one family at least, a single point mutation that inactivates or drastically lowers the catalytic activity of FMO3 results in trimethylaminuria. The likelihood of this is increased by the fact that Pro-153 appears to be a highly conserved residue among not just the mammalian FMOs but also other eukaryotic and prokaryotic
xenobiotic-metabolizing flavoproteins. Pro-153 is the first residue of the ProXxxXxxPro motif that is located between the FAD- and NADPH-pyrophosphate-binding domains (33 residues to the N-terminal side of the NADPH-binding domain) of all known mammalian FMO sequences bar FMO5. The reader will recall that FMO5, which contains an alanine at position 153, is unable to catalyze the metabolism of substrates common to the other known forms. The same motif is also located at the same relative position between the FAD- and NADPH-binding domains of bacterial cyclohexane monooxygenase and several homodimer flavoprotein disulphide oxidoreductases, for example, glutathione reductase, mercuric reductase and trypanothione reductase. Final proof, however, that the Pro-153 to Leu-153 mutation is capable of inducing trimethylaminuria requires the study of other affected families and, preferably, heterologous expression of the mutant FMO3 protein to determine if catalytic activity really is affected by this mutation. Note: In a personal communication with I.R. Phillips just prior to publication of this thesis, I was informed that heterologous expression of the mutant FMO3 protein has indeed been achieved. No catalytic activity (as measured by S-oxidation of methimazole) was detected, so it would appear that the Pro-153 to Leu-153 mutation is responsible for trimethylaminuria in the case of at least one family.

It would seem possible that the differing degrees of severity of trimethylaminuria could result from differences in the nature of genetic mutation affecting the FMO gene primarily responsible for TMA N-oxidation; for example, those with the more mild conditions might have a point mutation in the coding sequence that lowers enzyme activity or a mutation in the promoter region lowering the rate of transcription, whereas more severe cases might be due to mutations that completely inactivate the enzyme or abolish expression altogether. In any case, one would expect trimethylaminuria sufferers to display a reduced ability to monooxygenate other compounds known to be substrates for FMO such as nicotine, dimethylamphetamine, imipramine, nicotinamide, guanethidine, metyrapone or pinacidil. In connection with this, it has been shown that two siblings with trimethylaminuria failed to form nicotine N-oxide upon challenge with nicotine, suggesting co-segregation for these two defects (288). In another study (300), an association between trimethylaminuria and an inability to N-dealkylate verapamil was not established. If FMO3 is the gene responsible for trimethylaminuria, and consequently FMO3 activity is partially or completely deficient in individuals with the condition, it is interesting to speculate what consequences this might have on their long-term susceptibility to various clinical conditions. For example, FMO3 has been demonstrated to be the major FMO form responsible for methionine sulphoxidation (27).
1.11ii Other conditions
It is now believed that FMOs play an important role in the metabolism of the triphenylethylene antioestrogenic-anticancer therapeutic agent, tamoxifen (TXF). TXF is a major drug used in the treatment of breast cancer. Its antioestrogenic properties prompted its development as a therapeutic agent for hormone responsive (oestrogen receptor-positive) breast tumours (301, 302). Unexpectedly, a considerable number of patients with oestrogen-negative tumours also respond to tamoxifen therapy (303). It is also being considered for prophylactic use in women considered at high risk of breast cancer (304). However, of some concern is the observation that TXF treatment appears to increase the risk of endometrial cancer in humans and induces hepatocellular carcinoma in rats (305-308). It is thought that the metabolism of TXF may contribute to its antioestrogenic, anticancer and carcinogenic properties, and may also play a role in the development of tumour resistance in patients on long-term TXF therapy. Tamoxifen is metabolized by liver microsomes from animals and humans into a variety of compounds, most notably TXF N-oxide, N-desmethyl-TXF and 4-hydroxy-TXF (309-313). Of these three major metabolites, the cytochromes P450 have been demonstrated responsible for the formation of N-desmethyl-TXF and 4-hydroxy-TXF (309, 314), whereas FMO has been found to catalyze formation of TXF N-oxide (315). Evidence in the case of the latter includes the observations that mild heat treatment of liver microsomes (50°C for 90s) and addition of methimazole both inhibit N-oxidation of TXF (316). Furthermore, incubations of TXF with purified mouse liver FMO results only in TXF N-oxide formation (309). Of possible significance with regards to the negative side-effects of TXF therapy is the finding that TXF undergoes metabolic activation by both cytochromes P450 and FMO to a reactive intermediate that binds irreversibly to proteins and DNA (221).

As has been mentioned many times previously, methimazole is a classic FMO substrate. Methimazole is a drug administered to humans in the treatment of hyperthyroid conditions and belongs to the thioureylene class of antithyroid drugs, specifically, the thioamides. Recently published experiments with rats administered methimazole indicate that a reactive intermediate generated in the course of methimazole S-oxidation by FMO is an olfactory system toxicant (317). Large doses (300mg/kg/d i.p. as opposed to the standard human oral dosage of 0.2-2mg/kg/d) administered to Long-Evans rats were shown to cause almost complete destruction of the olfactory epithelium. Dose-response analysis further demonstrated that these rats experienced detectable olfactory mucosal damage at dosages as low as 25mg/kg/d i.p. and 50mg/kg/d when administered orally. It remains to be seen whether the amounts of methimazole and related
drugs taken by humans are capable of causing any olfactory damage in the long-term.

We have already discussed (this Section, Subsection 1.11i: Fish-odour syndrome/Trimethylaminuria, and Section 1.4: FMO activity in relation to endocrine and nutritional status) that there is ample evidence for the fact that the nature of the diet can have significant effects on FMO activity. For example, it was mentioned how progoitrin (a thioglucoside found within Brassica species such as cabbage, cauliflower, brussel sprouts, broccoli and swede that undergoes conversion by intestinal bacteria to the potent goitrogen goitrin) is a known inhibitor of FMO activity (272), although the evidence suggests that even excessive consumption of progoitrin-containing food items does not significantly retard N-oxidation of the classic FMO substrate, trimethylamine (274, 275). Another plant component that may modulate FMO activity is indole-3-carbinol (I3C), an alkaloid that is also found in significant concentrations within vegetables of the Brassica variety. I3C has been shown to provide chemoprevention against the development of both spontaneous and chemically induced cancers in numerous animal models (318-320). This has lead to the suggestion that it may be one of the components of a diet high in vegetable content that epidemiological studies suggest are correlated with a reduced risk of cancer in humans. Human clinical trials have been undertaken to evaluate the effectiveness of I3C in the chemoprevention of breast cancer, endometrial cancer and other oestrogen-related cancers (321-323). However, it must be noted that under certain experimental conditions, I3C can elicit promotional effects leading to increases in both tumour incidence and formation of neoplastic lesions (324-326). I3C is a relatively unstable compound that has been shown to undergo a series of condensation reactions in the acid environment of the stomach, leading to the formation of various dimers, linear and cyclic trimers and tetramers, in addition to some high molecular mass oligomers (327). A number of these condensation products are Ah receptor agonists, providing an explanation for the relative potency of orally administered I3C in the induction of both Phase I and Phase II enzymes, such as the cytochrome P450 enzymes CYP1A1, CYP1A2, CYP2B1/2 and CYP3A1/2 (328, 329). I3C induction of cytochrome P450 enzymes has been show to result in altered metabolism of steroid hormones, for example, in the CYP1A2-dependent upregulation of the 2-hydroxylation of oestrogens (328), and provides a rationale for the use of I3C in the chemoprevention of oestrogen-dependent tumours (321-323). Of relevance to this thesis is a report by Larsen-Su et al. (330) that dietary I3C dramatically inhibits both the expression (protein amounts) and activity (thiourea-dependent S-oxidation of thiocholine
hydrochloride) of FMO1 in both the liver and intestine of male Fischer rats. The inhibition showed both a time and dose dependency, resulting in an eight-fold reduction in FMO1 expression in liver and almost total ablation of FMO1 in intestinal tissues at higher I3C dosages. The expected hepatic induction of CYP1A1 amounts in these treated animals was also demonstrated. Thus, the net result of dietary I3C administration to these rats was a marked shift in amounts of monooxygenase, from FMO to cytochrome P450. It would seem logical that such a shift could lead to a dramatic alteration in the metabolism, disposition and toxicity of a number of xenobiotics, and that testing to see if I3C has similar effects in humans before widespread administration would seem a prudent measure.

FMOs are known to be sensitive to post-mortem thermal inactivation and anaerobiosis. For example, Kaderlik and Ziegler have shown that removal of molecular oxygen from FMO1 preparations, especially in the absence of NADPH, results in an irreversible and precipitous loss of enzyme activity even at ambient temperature (147). Thus, it may be that certain disease states such as ischaemic heart disease, ischaemic bowel disease or other conditions where oxygen supply to a tissue is restricted such as strokes and some surgical procedures, may result in a significant loss of FMO activity. Even a temporary decline in FMO activity could conceivably have consequences for the tissue concerned: an impact on the ability to metabolize drugs and maintain normal cellular homeostasis are examples that come to mind. Alternatively, it could be argued that a loss of FMO activity precipitated by ischaemic conditions could decrease the propensity of the cell to form sulphenic acids from dietary foodstuffs, leading to the preservation of cellular NADPH stores and preventing the formation of reactive sulphine metabolites.

Other examples of disease states that could impact on FMO activity are not difficult to think of, although what effect this would have on the progress of the disease is highly speculative. It could be postulated, for instance, that in conditions such as AIDS, whereby the structure and function of the intestinal villi disappear, the patient in question would be compromised with regards to the normal absorption and metabolism of chemicals, drugs and nutrients. As it seems likely the intestine is a significant site of FMO action (330), the AIDS patient would probably be at a significant disadvantage with respect to the normal, protective processes of xenobiotic oxidation by FMO.
1.12 The Scope of this thesis

In Chapter 3 (Localization of human FMO2 and FMO5 genes to chromosome 1q), I shall describe the outcome of my attempts to identify the chromosomal location of the human FMO2 and FMO5 genes, through the screening of human-rodent somatic cell hybrids by PCR. Prior to undertaking this work, the other three known human FMO genes (FMO1, FMO3 and FMO4) had already been localized to the long arm of chromosome 1 (1q) using similar methods. It was intended that the results of this work would assist in determining whether the entire human FMO gene family is clustered on 1q.

During the course of my research, I made numerous attempts to isolate human genomic DNA fragments containing FMO genes. It was intended that the isolation of such fragments would allow further studies into the molecular basis of how FMO gene expression is regulated, as presumably 5' flanking promoter sequences would also be obtained. Furthermore, it was believed that these genomic fragments would allow a rapid determination of the relative order of the human FMO genes on 1q. In Chapter 4 (Isolation of YACs bearing FMO-containing human genomic inserts), an account is given of how I successfully isolated human genomic DNA fragments containing FMO genes by screening a human genomic DNA library constructed in yeast artificial chromosomes (YACs). The results of Southern hybridization studies on the YAC clones isolated from this library (also given in Chapter 4) allowed me to determine which FMO genes were present within the respective clones. They also allowed me to speculate on the possible order of the human FMO genes on 1q. By then going on to perform fluorescence in-situ hybridization (FISH) analysis of human metaphase chromosomes with these YAC clones (Chapter 5: Fluorescence in-situ hybridization analysis of YAC clones bearing human genomic sequences containing FMO genes), I was able to further refine the genomic location of each of the five FMO genes. Chapter 6 (Pulsed field gel electrophoresis of YAC clones bearing human FMO sequences) describes how, through employment of pulsed field gel electrophoresis, I was able to determine the sizes of the genomic inserts of three of these clones.

Chapter 7 (Screening of a human chromosome 1-specific cosmid library for FMO genes) details another one of my attempts to isolate human genomic DNA fragments containing FMO genes. On this occasion, I screened a human chromosome 1-specific genomic DNA library constructed in cosmids. The results of the Southern hybridization experiments performed on the cosmid clones selected from this library turned out to be harder to interpret than for the YAC
clones in Chapter 4. Nevertheless, I went on to perform FISH analysis with the more promising of these clones, the results of which I describe in Chapter 8 (Analysis of cosmid clones isolated from a chromosome 1-specific library by fluorescence in-situ hybridization analysis).
Chapter 2

Materials and methods
2.1 Chapter 3: Localization of human *FMO2* and *FMO5* genes to chromosome 1q

### 2.1i *FMO2* PCR primers

Oligonucleotides were synthesized on a PCR-MATE DNA synthesizer (model 391, Applied Biosystems) from the human *FMO2* cDNA sequence as determined by C.T. Dolphin and were designed to anneal to sequences located on either side of the translation termination codon. Sequences as follows:

5’ CTCCTATTAGTATCGCCTGGTTGG 3’, and
5’ TACTGGATCCTGACAAGATAATAAAGCCCAAAG 3’.

Use of these primers in a polymerase chain reaction (PCR) was originally predicted to result in the amplification of a DNA fragment 252bp in size. This was previously confirmed through the use of genomic DNA or the full-length human *FMO2* cDNA as template. Use of *FMO1*, *FMO3*, *FMO4* or *FMO5* cDNA sequences as templates failed to generate an amplification product, thereby confirming the specificity of these primers for the presence of *FMO2*.

### 2.1ii *FMO5* PCR primers

As for *FMO2* primers, *FMO5* oligonucleotides were synthesized on a PCR-MATE DNA synthesizer (model 391, Applied Biosystems). Primer sequences were designed from the human *FMO5* cDNA sequence as determined by C.T. Dolphin and were designed to anneal entirely within the 3’-untranslated region. Sequences as follows:

5’ CTCTCAGTTTCATATTGCCCAG 3’, and
5’ ACATTATTTCTTTATCTCTCAGG 3’.

When using human genomic DNA or human *FMO5* cDNA as template, these primers were previously shown to successfully amplify the predicted 403bp DNA fragment. Specificity for *FMO5* was demonstrated by a failure to direct amplification from *FMO1, FMO2, FMO3,* or *FMO4* human cDNA templates.

### 2.1iii Human-rodent somatic cell hybrids

The fifteen somatic cell lines referred to in this thesis were a mixture of human-mouse and human-hamster hybrids (See Chapter 3, Table 3.1) and were kindly provided by Sue Povey in the form of genomic DNA preparations (prepared by the method of Edwards *et al* (331)). All have been described and characterized previously (See Chapter 3, Tables 3.1 and 3.2) by a mixture of isoenzyme
analysis (332) and karyotyping, although some hybrids have been cloned and recharacterized since.

2.1iv Genomic DNA Preparation

Human genomic DNA was prepared from whole blood according to the method described by Lahiri and Nurnberger (333). Specifically;

• 10 ml whole blood was drawn with a sterile syringe.

• Immediately, the blood was transferred to a 30 ml Sterilin Universal tube containing 200 μl of 15% EDTA.

• 10 ml of the low salt buffer TKM1 (10 mM Tris-HCl, 10 mM KCl, 10 mM MgCl₂, 2 mM EDTA, pH 7.6) was added to the collected blood.

• The cells were then lysed by addition of 250 μl of Nonidet P-40 (Sigma) and centrifuged at 2200 rpm for ten minutes at room temperature in an MSE Centaur 2 centrifuge.

• The supernatant was discarded and the pellet resuspended with 10 ml of TKM1 buffer.

• Centrifugation was repeated as before.

• After discarding the supernatant once more, the pellet was gently resuspended in 1.6 ml of the high salt buffer TKM2 (10 mM Tris-HCl, 10 mM KCl, 10 mM MgCl₂, 0.4 M NaCl, 2 mM EDTA, pH 7.6).

• 100 μl of 10% SDS was then added and the whole suspension mixed thoroughly by pipetting back and forth several times before incubating for ten minutes in a waterbath at 55 °C.

• Following addition of 600 μl of 6 M NaCl, the mixture was partitioned into two 1.5 ml Eppendorf centrifuge tubes. Both tubes were centrifuged at 13 000 rpm in an Eppendorf centrifuge for five minutes.

• At this stage, the supernatants were transferred to fresh Eppendorf tubes and the protein pellets discarded.
• To precipitate the DNA, two volumes of absolute ethanol were added and the tubes inverted a few times until the DNA became clearly visible.

• Using a glass Pasteur pipette, the precipitated DNA strands were fished out and transferred to fresh Eppendorf tubes containing 1 ml of ice-cold 70% ethanol.

• DNA was then pelleted by centrifugation for five minutes at 13 000 rpm in an Eppendorf centrifuge. The supernatant was poured off and the tube air-dried.

• Finally, the DNA pellet was resuspended in 0.5 ml TE buffer, pH 8.0 and placed in a water bath at 65°C for fifteen minutes before storage at 4°C.

• DNA yield was measured by absorbance at wavelength 260 nm in a spectrophotometer (multiplication of A260 by 50 and the dilution factor gives μg/ml DNA). Integrity (degree of shearing/degradation) of the genomic DNA was checked by running a 0.6% agarose (Sigma)-1×TBE gel (1×TBE: 90 mM Tris-HCl, 90 mM boric acid, 2 mM EDTA, pH 8) containing 0.5 ng/ml ethidium bromide (BDH Merck).

Mouse and hamster genomic DNA were kindly provided by Sue Povey.

2.1v FMO2 PCR reactions
After trial-and-error to optimize the ideal reactant concentrations and PCR conditions, each reaction was made up to a final volume of 40 μl and contained the following of each;

Template: 1 μg mouse, hamster or human genomic DNA, or 2 μg somatic cell hybrid DNA.

Primers: 50 pmol of each primer.

dNTPs: 50 μM final concentration of dATP, dCTP, dTTP and dGTP (Pharmacia), respectively.

Polymerase enzyme: 2.5 units BIOTAQ DNA polymerase (Bioline, UK)*.

Buffer: 10×NH4 Buffer (Bioline, UK) [160 mM (NH4)2SO4, 670 mM Tris-HCl pH 8.8, 0.1% Tween-20], added to achieve 1× final concentration.

MgCl2: (50 mM, Bioline, UK), diluted to a final concentration of 2 mM.
**H2O**: Double-distilled to 40µl.

*Added last after a two minute, 94°C ‘hot start’.

**Note**: To overcome DNA contamination problems, PCR buffer, H2O, MgCl2, pipette tips and reaction tubes were UV irradiated in a Stratagene UV Stratalinker prior to use.

Reactions were overlaid with an equal volume of mineral oil and amplified in a thermal reactor (Hybaid), programmed as follows:

94°C for 1min;  
57°C for 1min;  
72°C for 1min;  

for 36 cycles.

**2.1vi FM05 PCR reactions**

Reactions were made up to a final volume of 40µl. Results were found to be optimal when reactions were made up as follows:

**Template**: 1µg mouse, hamster or human genomic DNA, or 2µg somatic cell hybrid DNA.

**Primers**: 50pmol of each primer.

**dNTPs**: 300µM final concentration of dATP, dCTP, dTTP and dGTP, respectively (Pharmacia).

**Polymerase enzyme**: 2.5 units BIOTAQ DNA polymerase (Bioline, UK)*.

**Buffer**: 10xNH4 Buffer (Bioline, UK), diluted to 1x final concentration.

**MgCl2**: (50mM, Bioline, UK), diluted to a final concentration of 2mM.

**H2O**: Double-distilled to 40µl.

*Added last after a two minute, 94°C ‘hot start’.
Note: As for the FMO2 PCR reactions, problems with DNA contamination were eliminated by UV irradiation of PCR buffer, H2O, MgCl2, pipette tips and reaction tubes in a Stratagene UV Stratalinker prior to use.

Reactions were overlaid with an equal volume of mineral oil and amplified in a thermal reactor (Hybaid), programmed as follows:

94°C for 1min;
45°C for 1min;
72°C for 1min;

for 36 cycles.

2.1vii Agarose gel electrophoresis
Completed PCR reactions were separated through a 2% agarose-1xTBE gel containing 0.5ng/ml ethidium bromide. DNA samples to be loaded on the gel had an appropriate amount of 10x loading dye (10ml of 10x loading dye: 25mg bromophenol blue, 4ml glycerol, 6ml H2O) added. Molecular weight standards consisted of 1-kb ladder (Gibco-BRL Life Technologies). After separation, DNA bands were visualized by placing of the agarose gel on a UV transilluminator.

2.2 Chapter 4: Isolation of YACs bearing FMO-containing human genomic inserts

2.2i ICRF YAC human genomic DNA library
The ICRF YAC human genomic DNA library was provided by the UK HGMP Resource Centre, Hinxton Hall, Cambridgeshire and has been described previously (334). It comprises 20,500 YAC clones in total and gives coverage of three human genomes: a human female lymphoblastoid (48XXXX) cell line, a human male lymphoblastoid (49XYYYY) cell line and a human female Huntington disease lymphoblastoid (HD1, 46XX) cell line. Clones are spotted in an ordered grid formation across fourteen nylon filters. On each filter, up to sixteen microtiter plates, or 1536 clones are spotted. A clone deemed suitable for selection following screening is easily identifiable by the coordinates of the grid system.
2.2ii Prehybridization of YAC library nylon filters

Prehybridization was for a minimum of six hours at 60°C in a Stratagene Hybridizer 600 with the following prehybridization solution:

- 6xSSPE, diluted from 20x stock (20x stock: 3M NaCl, 0.02M EDTA, 0.2M Na$_2$HPO$_4$).

- 5xDenhardt’s solution, diluted from 50x stock (50x Denhardt’s solution: 1% bovine serum albumin [Miles Scientific], 1% ficoll 400 [Sigma], 1% polyvinylpyrrolodine [Sigma]).

- 0.5% sodium dodecyl sulphate (Sigma)

- 10% dextran sulphate (Sigma)

- 50μg/ml denatured salmon sperm DNA (Sigma)*

- Volumes were made up with double-distilled H$_2$O and warmed to dissolve the dextran sulphate, before passing through a 50μm pore-size filter.

*Denatured salmon sperm DNA was made as follows:

- Salmon sperm DNA (Sigma) was dissolved to a concentration of 10mg/ml.

- NaCl was added to a concentration of 0.1M.

- DNA was extracted once with an equal volume of buffered (pH 8.0) phenol/chloroform (1:1).

- DNA extract was then sheared by drawing back-and-forth through a 17 gauge needle at least twelve times.

- Sheared DNA was precipitated by addition of two volumes of ethanol and recovered by centrifugation. The DNA pellet, once dried, was dissolved by addition of H$_2$O to a final concentration of approximately 10mg/ml (estimated by measurement of optical density at wavelength 260nm with a spectrophotometer: multiplication by 50 and the dilution factor gave μg/ml DNA concentration). Immediately prior to use, the sheared salmon sperm DNA was boiled for five minutes and chilled on ice.
2.2iii Preparation of FMO cDNA probes

The full-length cDNAs of FMO1, FMO2, FMO3, FMO4 and FMO5 were provided as clones courtesy of C.T. Dolphin, with JM101 E.coli the host for the FMO1, FMO3 and FMO4 clones and DH5 alpha E.coli the host for the FMO2 and FMO5 clones. The cDNAs for FMO1, FMO3 and FMO4 have been described by Dolphin et al previously (84, 104, 133). The vectors of the respective cDNA clones were as follows: FMO1 and FMO5 cDNAs were ligated into pUC19, and FMO2, FMO3, and FMO4 cDNAs were ligated into pBS (Bluescript). Clones were received as spreads on agar plates with added ampicillin antibiotic.

The first step undertaken was to convert the host of the FMO1, FMO3 and FMO4 cDNA construct clones from JM101 to JM109. This was undertaken as follows;

(a) Plasmid construct for the FMO1, FMO3 and FMO4 cDNA clones was isolated by a method based on a procedure first described by Birnboim and Doly (335) and subsequently modified by Ish-Horowicz and Burke (336). Protocol as follows;

•Individual colonies were picked from the respective agar plates provided by Dolphin and used to inoculate vials containing Luria Bertani (LB) broth with added ampicillin (50μg/ml). Inoculated vials were left shaking overnight in a waterbath at 37°C.

•Cells from the resultant cultures were harvested by centrifugation for one minute in an Eppendorf centrifuge after transfer to 1.5ml Eppendorf tubes.

•Cell pellets were resuspended in 100μl of ice-cold ‘Solution 1’ (50mM glucose, 10mM EDTA, 25mM Tris-HCl, pH 8.0) and left for five minutes at room temperature.

•200μl of ‘Solution 2’ (200nm NaOH, 1% SDS) was added and the tubes transferred to ice for five minutes.

•Addition of 150μl of ice-cold ‘Solution 3’ (3M potassium acetate, 5M ethanoic acid) followed. At this point, the tubes were briefly vortexed before placing on ice for a further five minutes.
• After centrifugation for five minutes in an Eppendorf centrifuge, the supernatants were transferred to fresh Eppendorf tubes. To eliminate RNA, each tube had RNase A (Sigma) added to a final concentration of 20\(\mu\)g/ml (from a boiled 10mg/ml stock) before incubation at 37°C for twenty minutes.

• An equal volume of buffered (pH 8) phenol/chloroform (1:1) was transferred to each tube and the mixtures briefly vortexed. Aqueous and organic phases were separated by centrifugation in an Eppendorf centrifuge for just two minutes. Carefully, the upper aqueous phases only were drawn and transferred to fresh Eppendorf tubes.

• Plasmid DNA was precipitated by addition of 2.5 volumes of ethanol to each tube and storage on dry ice (solid CO\(_2\)) for at least ten minutes. After centrifugation in an Eppendorf centrifuge for ten minutes, the supernatants were carefully removed and the DNA pellets resuspended with 70% ethanol. Centrifugation was then repeated as before. This time, after careful removal of the supernatants, the pellets were left to dry.

• Finally, the DNA pellets were dissolved by addition of TE solution (10mM Tris-HCl, 1mM EDTA, pH 8). The DNA concentrations of the preparations were measured by optical density at wavelength 260nm, as described above.

(b) The respective plasmid preparations were then used to transform competent JM109 cells. The processes of generating competent JM109 cells and the subsequent transformations were based on the method described by Sambrook et al. (337) and were performed as follows:

• Using a sterile platinum wire, JM109 cells from a frozen stock were streaked across a SOB agar plate, and colonies left to appear by incubation at 37°C overnight.

• Well-isolated colonies from the agar plate were picked and grown by shaking in SOB + 20mM MgSO\(_4\) medium (BIO101 SOB capsules) at 37°C.

• Cell growth over 2.5-3hrs was regularly monitored by checking optical density at wavelength 600nm and was not allowed to exceed 10^8 cells/ml.

• Cells were transferred aseptically to ice-cold 50ml tubes (Falcon 2070) and placed on ice for ten minutes.
• Harvesting of cells took place by centrifugation (MSE Centaur 2) at 4000 rpm (4°C) for ten minutes.

• Supernatants were discarded and the bacterial pellets resuspended in 20 ml of ice-cold FSB solution (10 mM potassium acetate, 45 mM MnCl₂, 10 mM CaCl₂, 100 mM KCl, 3 mM hexaminecobalt chloride, 10% glycerol). Tubes were left standing in ice for ten minutes.

• Cells were recovered by centrifugation as before and resuspended in 4 ml of ice-cold FSB solution.

• To the FSB cell suspensions were added 140 μl of dimethyl sulphoxide (DMSO), before placing the tubes back on ice for fifteen minutes. After this, a further 140 μl of DMSO was added.

• Immediate transfer of 50 μl of the cell suspensions to chilled, sterile Eppendorf tubes, followed by snap-freezing in liquid nitrogen, provided competent cells that could be stored at -70°C until needed.

• Aliquots of JM109 competent cells prepared as just described were thawed on ice.

• The entire 50 μl of competent cells in each Eppendorf tube was transferred to chilled, sterile polypropylene tubes (Falcon 2059, 17 mm x 100 mm), which in turn were placed in ice.

• An estimated 1 ng of plasmid DNA (in this case, from the FMO1, FMO3 and FMO4 preparations obtained as described above) was added to each tube of competent cells. Tubes were left in ice for 30 minutes.

• 'Heat-shock' was performed by placing the tubes in a waterbath set to 42°C.

• After exactly 90 seconds in the waterbath, the tubes were transferred back to ice for 1-2 minutes.

• 800 μl of SOC medium (SOB medium + 20 mM glucose) was then added to each of the tubes, which were subsequently removed from the ice and placed in a shaking incubator/waterbath set to 37°C. The cells were left shaking for 45 minutes to allow their recovery and expression of the antibiotic resistance marker encoded by the plasmid.
• Cell cultures were spread on SOB agar + 20mM MgSO$_4$ plates (200µl per 90mm plate) with added ampicillin (50µg/ml).

• After drying, the plates were left to incubate at 37°C overnight.

• Individual colonies from the plates were picked and transferred to sterile Eppendorf tubes containing 850µl LB medium (50µg/ml ampicillin) and 150µl glycerol. Tubes were snap-frozen by immersion in liquid nitrogen to create glycerol stocks for storage at -70°C.

(c) The veracity of the transformants was checked by repeating the steps described above in (a). In other words, JM109 transformants were grown from the respective glycerol stocks and plasmid DNA isolated. Aliquots from these plasmid preparations were digested with a range of restriction enzymes and the outcome determined by separation of the digests through a TBE-0.8% agarose gel. In each case, the pattern of DNA fragments obtained was indicative of the full length cDNAs being present for \( FMO1, FMO3 \) and \( FMO4 \), respectively.

Further \( FMO1, FMO3 \) and \( FMO4 \) plasmid preparations were performed. In addition, identical procedures were used to obtain plasmid preparations containing the \( FMO2 \) and \( FMO5 \) cDNA clones, which had been taken directly as colonies from the agar plates provided by Dolphin and stored as glycerol stocks in the manner previously described.

It was considered preferable to screen the YAC human genomic DNA library with just the full-length \( FMO \) cDNAs, rather than cDNA plus plasmid vector. Thus, it was necessary to use restriction enzymes to liberate cDNA insert from vector. The digest performed for each \( FMO \) construct is considered in turn below.

\( FMO1: FMO1 \) cDNA had been ligated into the EcoR1 cutting site of pUC19 and does not contain an internal EcoR1 restriction enzyme cutting site. It was therefore liberated by digesting the construct with EcoR1 enzyme (Pharmacia).

\( FMO2: \) Digestion with the two restriction enzymes Xba1 (Pharmacia) and BamH1 (Pharmacia) cleaved the \( FMO2 \) cDNA intact from its pBS vector.

\( FMO3: \) \( FMO3 \) cDNA was liberated entirely from its pBS vector by digestion with BamH1 and Pst1 (Pharmacia).
**FM04:** Cutting out the FM04 cDNA intact from its pUC19 vector was relatively complicated owing to the loss of one of the EcoR1 cutting sites used to ligate the construct originally, and by the fact that this cDNA contains an internal EcoR1 cutting site. Using the MacVector computer programme, it was calculated that digestion with the two restriction enzymes Bgl2 (Pharmacia) and Ava3 (Gibco-BRL) would liberate all but 100 base pairs from each end of the FM04 cDNA. As the two respective restriction enzymes were from different manufacturers and, therefore, required the use of separate buffers provided by those manufacturers (One-Phor-All from Pharmacia and React 3 from Gibco-BRL), two sequential reactions had to be performed, with an ethanol-induced DNA precipitation required in between.

**FM05:** The cDNA was cut free from its pBS vector by digestion with the restriction enzymes Xbal (Pharmacia) and Pst1 (Pharmacia).

All of the above digests contained approximately 6μg plasmid DNA and were checked for successful completion by running small amounts of the digests (approximately 200ng) on a 0.8% TBE-agarose gel. After checking, the remainder of the digests were loaded on a 0.8% TAE-low melting point (LMP) agarose (BDH Merck) gel (TAE: 40mM Tris-acetate, 5mM sodium acetate, 1mM EDTA, pH 8.0) and separated. The method chosen for isolating just the cDNA inserts from the gel for use in radioactive labelling was based on that described by Feinberg and Volgestein (338). After separation of the DNA fragments in the LMP gel had taken place, the fragments were viewed with a hand-held UV light source (to reduce the UV dose that would accompany the use of a UV transilluminator and subsequent DNA damage) and the desired bands corresponding to the cDNA inserts identified by virtue of size. Using a clean scalpel, these bands were neatly excised from the gel and removed. The agarose blocks were then transferred to preweighed 1.5ml Eppendorf tubes. The tubes plus agarose were weighed and the mass of agarose calculated thereof. Double-distilled H2O was added to the tubes at a ratio of 3ml H2O/g of agarose gel and the tubes placed in boiling water for seven minutes to dissolve the gel and denature the DNA. The DNA samples were then stored at -20°C, but were reboiled for three minutes prior to the taking of samples for labelling. The concentration of DNA in these preparations was estimated by running a range of volumes through an agarose gel alongside varying quantities of pUC19 DNA and visually comparing DNA band intensities in the gel. The estimate this provided could be compared to the predicted amount of DNA that would be present if all of the cDNA insert had been recovered from the 6μg of construct digested.
2.2iv Radiolabelling of FMO cDNAs
Radioactive labelling of the FMO cDNAs was achieved by the oligonucleotide random primer method. Labelling reactions consisted of the following:

- 9μl 20mM dithiothreitol (DTT) (Sigma)
- 6μl [α\textsuperscript{32}P]-dCTP at 10μCi/μl (i.e. 60μCi total) and 3000 Ci/mmol (NEN-Dupont)
- 2μl dNTP mix: 5mM concentration of dTTP, dATP & dGTP, respectively (Pharmacia)
- 9μl 10x Random primer (RP) buffer (900mM HEPES adjusted to pH6.6 with 4M NaOH, 100mM MgCl\textsubscript{2})
- 100ng FMO cDNA in LMP agarose, prepared as described above*
- 1.5μl Random hexadeoxyribonucleotide primers (Sigma), previously diluted in TE buffer to 90 OD units/ml*
- Double-distilled H\textsubscript{2}O to a final volume of 90μl*
- 1μl (10 units) Klenow fragment of E. coli DNA polymerase I (Pharmacia)**

*It was found that by boiling just the cDNA, random primers and H\textsubscript{2}O together for five minutes and chilling on ice, before adding to the DTT, dNTPs, [α\textsuperscript{32}P]-dCTP and RP buffer, labelling efficiency could be significantly improved.

**Klenow polymerase was added last of all.

After mixing the constituents of the reaction, incubation proceeded for a minimum of one hour at 37°C, or three hours if left at room temperature.

To allow calculation of the specific activity of the cDNA probes, 1μl samples of the completed labelling reactions were spotted on two separate pieces of Whatmann DE81 paper. After allowing the disks to dry, one of them was repeatedly washed in 0.5M Na\textsubscript{2}HPO\textsubscript{4} to remove unincorporated [α\textsuperscript{32}P]-dCTP and was then dried. The two disks (unwashed total radioactive counts and washed incorporated radioactive counts) were then placed in separate scintillation vials containing Ecosint A (National Diagnostics). Radioactive
counts as counts per minute (cpm) were measured in a scintillation counter (Phillips 4700). The ratio between the two filters gave a figure for percentage incorporation of radionucleotide, whereas from the cpm reading of the washed filter it was possible to calculate the specific activity of the probe as cpm/µg DNA. A probe was considered ‘hot’ enough for use in Southern hybridization experiments if its specific activity was in excess of 10^8 cpm/µg DNA, at the very minimum. All probes used in the Southern hybridization experiments described in this thesis were of specific activity greater than or equal to 5x10^8 cpm/µg DNA.

Following completion of the labelling reactions and measurement of probe specific activities, unincorporated nucleotides were removed by the use of Chromaspin+TE -10 LC columns (Clontech Lab Inc.). Prior to loading on the Chromaspin columns, the nucleic acid concentrations of the labelling reactions were brought up to the manufacturers’ specification for optimal probe recovery (approximately 60µg/ml) by the addition of appropriate amounts of tRNA. After pre-spinning of the chromaspin columns (3000 rpm in an MSE Centaur 2 centrifuge for five minutes), the reaction samples were carefully loaded. A second identical centrifugation step resulted in elution of the cDNA probes.

2.2. v Probe hybridization to YAC library nylon filters
Following prehybridization of the filters (see above), the prehybridization fluid was discarded. It was replaced by fresh prehybridization fluid to which had been added the five radiolabelled FMO cDNA probes (probe concentration was approximately 7ng/ml hybridization fluid for each probe). Just prior to adding, the probe were boiled for five minutes and instantly chilled on ice to render them single-stranded. Hybridization was left to proceed at 60°C in the hybridization oven overnight.

2.2. vi Washing of YAC library nylon filters
Following overnight probe hybridization, the filters were washed while shaking in the following manner:

- Twenty minutes at room temperature in 0.1xSSPE, 1% SDS
- Repeat
- Twenty minutes at 65°C in 0.1xSSPE, 1% SDS
- Repeat

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Filters were then briefly rinsed in cold 0.1xSSPE, drip-dried and wrapped in clingfilm (Saran wrap).

2.2vii Autoradiography
Washed filters in Saran wrap were placed inside cassettes with intensifying screens against X-ray film. The cassettes were placed at -70°C and the films left to expose overnight initially before development.

2.2viii Preliminary characterization of requested YAC clones
YAC clones requested from the primary screening of the library were received as stabs on AHC agar and so presented as red colonies (see below for a discussion on AHC medium/agar and why the yeast cells were red in colour). Immediately, glycerol stocks were made for each of the clones by direct transfer from the agar to Eppendorf tubes containing 200µl glycerol and 850µl YPD medium, before snap freezing in liquid nitrogen and storage at -70°C. YPD is an enriched all-purpose yeast growth medium (339) in which yeast strains, with or without YACs, grow vigorously. YPD is made up as follows:

*10g yeast extract (Difco)

*20g peptone (Difco)

*Bring to one litre with H₂O and adjust pH to 5.8

*After autoclaving, add 50ml filter-sterilized 40% (w/v) glucose

Each of the clones were grown by inoculating from glycerol stocks into 10ml of AHC medium plus 50µg/ml ampicillin (to prevent growth of contaminating bacteria) in 30ml Sterilin Universal tubes. AHC is a complete medium for growing Saccharomyces cerevisiae strain AB1380 (the yeast strain used for this library) while selecting for the YAC vector used to construct this library, pYAC4, regardless of whether an insert is present or not (340). AHC medium is made up as follows:

*1.7g yeast nitrogen base without amino acids and (NH₄)₂SO₄ (Difco)

*5g (NH₄)₂SO₄ (Sigma)

*10g acid hydrolyzed casein (casamino acids) (Sigma)
• 20mg adenine hemisulphate (Sigma)

• Bring to one litre by addition of H$_2$O and adjust pH to 5.8

• After autoclaving, add 50ml filter-sterilized 40% (w/v) glucose

pYAC4 was first described by Burke et al in 1987 (341). The 6kb left arm of the vector contains $ARS_I$ (yeast origin of replication) and $CEN_4$ (centromere) sequences. This maintains the artificial chromosome as a linear molecule in yeast through proper replication and segregation during cell division. It also contains the marker gene $trpI$ (coding for phosphoribosylanthranilate isomerase), which is used for positive selection of this arm in a growth medium lacking tryptophan (for example, AHC medium). The 3.4kb right arm has the $ura_3$ gene (coding for orotidine-5'-phosphate decarboxylase), which allows for positive selection of the arm in a growth medium lacking nucleosides (again, an example of this would be AHC medium). Lying between the two arms is the $sup_4$ gene. The gene product of $sup_4$ is a mutant tyrosine tRNA which suppresses the ochre (nonsense) mutation in the $ade_2$ gene of the host. The EcoR1 cloning site of the vector is located within the $sup_4$ gene, so that when a fragment is cloned, the ochre suppression is eliminated. The consequent interruption in adenine metabolism results in the accumulation of the red pre-metabolite, phosphoribosylaminoimidazole, when levels of exogenous adenine are limiting, thereby providing recombinant clones with a characteristic red colour that can be used for selection. In AHC medium, the adenine is at slightly growth-limiting concentrations. As a result, when strain AB1380 is grown in AHC, red pigmentation is visible, but cell growth does not reach as great a density as when all-purpose, complex yeast growth mediums (such as YPD) is used. Finally, at each end of the artificial chromosome there are artificial telomeres, consisting of (C$_4$A$_2$) repeats derived from a Tetrahymena rDNA telomere. These sequences seed the formation of (C$_1$-3A) terminal repeats by yeast telomerase.

**Isolation of yeast genomic DNA**

Growth of the YAC clones in AHC was allowed to proceed overnight at 30°C in a shaking waterbath. The following genomic DNA protocol was then employed;

• Cells were harvested by centrifugation at 1000 rpm (MSE Centaur 2) for ten minutes.
• After decanting supernatant, cell pellets were resuspended in 0.5ml of yeast resuspension buffer (YRB) + 14mM β-mercaptoethanol. YRB: 1.2M sorbitol (Sigma), 10mM Tris-HCl, 20mM EDTA, pH7.5.

• Twenty units of lyticase enzyme (Sigma) was added to each tube and the cell suspensions incubated at 37°C until 80-90% of the cells were visible as spheroplasts under a microscope (this typically took about an hour and could be confirmed by the addition of water to the microscope slide: spheroplasts would immediately lyse).

• Spheroplast cell suspensions were transferred to 1.5ml Eppendorf tubes and centrifuged for one minute at 13 000 rpm in an Eppendorf microcentrifuge.

• The resultant cell pellets were resuspended in 0.5ml of 50mM Tris-HCl, 20mM EDTA, pH7.4.

• Addition of 50μl of 10% SDS was followed by incubation for 30 minutes in a water bath at 65°C.

• 200μl of 5M potassium acetate was then added and the tubes placed on ice for one hour.

• Tubes were then centrifuged for five minutes at 13 000 rpm in an Eppendorf microcentrifuge.

• The supernatant was retained and transferred to fresh Eppendorf tubes.

• An equal volume of isopropanol was added and the DNA left to precipitate at room temperature for five minutes.

• Tubes were then centrifuged in an Eppendorf microcentrifuge, but only very briefly (about ten seconds) to pellet the DNA. The supernatant was very carefully removed and the tubes left to air-dry.

• Once dry, the DNA was resuspended in 0.3ml TE solution (10mM Tris-HCl, 1mM EDTA, pH7.4).

• 15μl of a 1mg/ml solution of pancreatic RNase (Sigma) was added to each of the tubes and incubation left to proceed for 30 minutes at 37°C.
• Addition of 30μl of 3M sodium acetate followed.

• Two successive phenol/chloroform (1:1) extractions were then carried out.

• DNA precipitation was initiated by addition of two volumes of absolute ethanol. Tubes were placed at -70°C for ten minutes.

• Precipitated DNA was recovered by centrifugation at 13 000 rpm for ten minutes in an Eppendorf centrifuge.

• The supernatants were poured off and 1ml of 70% ethanol added to each tube.

• Tubes were again centrifuged at 13 000 rpm in an Eppendorf centrifuge for ten minutes.

• After pouring off the supernatants, tubes were left to air-dry.

• DNA in the tubes was resuspended in 100μl TE buffer and stored at -20°C.

*Restriction endonuclease digestion of yeast genomic DNA*

Genomic DNA preparations for each of the requested YAC clones were digested with EcoRI restriction endonuclease as follows:

• 6μg yeast genomic DNA

• 25 units EcoRI (Pharmacia)

• 6μl 10x One-Phor-All buffer (Pharmacia)

• Double-distilled H₂O to a volume of 30μl

Digestion was allowed to proceed for two hours at 37°C.

To check that digestion was complete, samples (2μl of reaction, plus 0.5μl 10x loading dye [10x loading dye: 25μg bromophenol blue, 4μl glycerol, 6μl H₂O]) underwent 0.8% agarose (Sigma) DNA gel electrophoresis in TBE (90mM Tris-HCl, 90mM boric acid, 2mM EDTA, pH 8) buffer. The gel contained 0.5ng/ml ethidium bromide.
Southern hybridization
Approximately 2μg of the yeast genomic DNA EcoRI digests were loaded on a 0.6% TBE-agarose gel containing 0.5ng/ml ethidium bromide. Radiolabelled molecular weight standards were prepared as follows;

• 5μl (approximately 1μg) 1kb ladder (Gibco-BRL Life Technologies)

• 6μl 5x T7 DNA polymerase buffer (200mM Tris-HCl, 50mM MgCl₂, 25mM DTT, 250μg/ml BSA [nuclease-free, Pharmacia])

• 4μl 2mM dGTP (Pharmacia)

• 4μl 2mM dTTP (Pharmacia)

• 4μl 2mM dCTP (Pharmacia)

• 3μl [α³⁵S]-dATP, 800 Ci/mmol & 10μCi/μl (NEN-Dupont)

• 10 units T7 DNA polymerase (Stratagene)

• Double-distilled H₂O to 30μl

The reaction was incubated for ten minutes in a 37°C waterbath before addition of 4μl 2mM dATP (Pharmacia) and a further ten minute incubation at 37°C. Storage was at -20°C.

After separation, the gel was photographed before beginning the following Southern blot protocol;

• The agarose gel was rinsed in distilled water.

• The gel was then covered in depurinating solution (0.25M HCl) for twenty minutes. During depurination, the gel was very gently agitated to avoid breaking.

• After rinsing in distilled water, the gel was covered in denaturation solution (1.5M NaCl, 0.5M NaOH) and gently agitated for 30 minutes.
• The gel was rinsed again in distilled water before being immersed in neutralization buffer (1.5M NaCl, 1M Tris-HCl, pH7.4) and gently agitated for thirty minutes.

• Meanwhile, a glass plate and large tray were used to construct a capillary transfer set-up. A wick made from two pieces of 3MM Whatmann paper was folded over the glass plate so that both ends of the paper touched the bottom of the tray. 20x SSC solution (3M NaCl, 0.3M trisodium citrate) was used to wet the wick and create a reservoir in the base of the tray.

• The treated gel was inverted and laid on top of the wick. A device, such as a plastic pipette, was used to gently roll-out any air bubbles.

• On top of the gel was carefully placed a piece of Electran Optimized Nylon Blotting Membrane (BDH Merck) cut to exactly the same size as the gel.

• Two sheets of Whatmann 3MM paper, also cut to the same size as the gel and wetted with 20x SSC solution, were placed on top of the nylon membrane.

• Ten sheets of Quickdraw (Sigma) blotting paper were placed on top of the 3MM paper. Finally, a glass plate was placed over the blotting paper and a small weight applied.

DNA transfer from the gel to the nylon membrane was usually left to proceed overnight, although it was found that after just one hour, most of the DNA transfer had already occurred. The collapse of the gel brought about by its dehydration during transfer, and the presence of loading dye on the nylon membrane was found to be indicative of a successful transfer. However, this was sometimes confirmed more precisely by re-staining the collapsed gel in ethidium bromide solution (1ng/ml) and checking for the presence of DNA bands on a UV transilluminator. Following successful transfer, DNA was covalently cross-linked to the nylon membrane by placing (DNA side upwards) inside a UV Stratalinker (Stratagene) with a piece of 3MM previously wetted with 20xSSC underneath, and choosing the ‘Auto Cross Link’ function (0.12 joules). The nylon membrane was then dried in an oven at 80°C and stored wrapped in Saran Wrap.
Prehybridization of Southern blot
Prehybridization took place in exactly the same manner as described above for the prehybridization of the ICRF YAC human genomic DNA library nylon filters.

Hybridization of the Southern blot
This was undertaken as described earlier for the hybridization of the ICRF YAC human genomic DNA nylon filters. FMO cDNA probes were radiolabelled and prepared in exactly the same fashion throughout.

Post-hybridization washing of the Southern blot nylon membrane
The same wash solutions and temperatures as were used for washing of the YAC human genomic DNA library nylon filters were employed.

 Autoradiography
Again, the procedures used were the same as described above for the YAC human genomic DNA library nylon filters.

2.2ix Assigning respective FMO genes to individual YAC clones
Agarose gel electrophoresis, Southern blots, radiolabelling of FMO cDNAs, prehybridization and hybridization of Southern blots, and autoradiography were all performed as described above. Washing of hybridized Southern blots were also identical, except that on occasions, where appropriate, additional high-stringency (0.1xSSPE, 1% SDS at 65-70°C) washes were performed to lower the strength of the signal (monitored through the use of a Geiger counter).

 Stripping of bound radiolabelled FMO cDNA probe
As some of the Southern blots were re-probed, it was necessary on such occasions to completely strip the nylon membrane of all probe bound. This was undertaken by immersion of the membrane in boiling 0.5% SDS solution, repeated until no radioactive signal could any longer be detected by use of a Geiger counter. Then, the film would undergo autoradiography to check that no exposure of X-ray film was possible.
2.3 Chapter 5: Fluorescence *in-situ* hybridization analysis of YAC clones bearing human genomic sequences containing FMO genes.

Fluorescence *in-situ* hybridization (FISH) analysis of YAC clones was undertaken with the kind assistance of Margaret Fox and Sue Povey. The protocol used was a modification of a technique first described by Pinkel *et al* in 1986 (342). Steps in the protocol were as follows;

*YAC clone DNA was isolated in the form of yeast genomic DNA preparations, in the manner described in Section 2.2viii (Preliminary characterization of requested YAC clones) above.*

*YAC DNA was labelled with biotin-14-dUTP by nick translation, using a Bionick kit (Gibco-BRL Life Technologies). Unincorporated nucleotides were removed using a Sephadex G-50 column.*

*Labelled DNA (100ng) was ethanol-precipitated, along with Cot-1-DNA (to suppress repetitive DNA elements) and herring sperm DNA (Sigma) (to act as carrier). After centrifugation to pellet the precipitated DNA, resuspension was performed with a hybridization solution (20μl) consisting of 50% formamide, 10% dextran sulphate and 2xSSPE (20xSSPE: 3.6M NaCl, 0.2M NaH₂PO₄, 0.02M EDTA, pH7.4), adjusted to pH 7.0.*

*Human metaphase chromosomes were obtained from lymphocyte cultures synchronized by addition of thymidine to block DNA synthesis. After removal of the block, 5-bromo-deoxyuridine (BrdU) was incorporated prior to harvest. Standard cytogenetic techniques were used for colcemid arrest, hypotonic treatment and fixing of the cells in methanol:acetic acid.*

*Slides were freshly prepared in the following manner before aging for two hours at room temperature prior to use;*  

- Slides were pretreated with RNase (100μg/ml in 2xSSC [20xSSC: 3M NaCl, 0.3M trisodium citrate]) under a coverslip in a moist chamber for one hour at 37°C.

- Slides were then washed with 2xSSC before dehydration through an alcohol series and air-drying.
- Incubation of the slides in proteinase K buffer (20mM Tris-HCl, 2mM CaCl$_2$, pH 7.4) for ten minutes was followed by a seven minute treatment with fresh proteinase K buffer containing proteinase K (50µg/ml) at 37°C.

- After proteinase K treatment, slides were washed in phosphate-buffered saline (PBS) (one litre of PBS is made as follows: 8g NaCl, 0.2g KCl, 1.44g Na$_2$HPO$_4$, 0.24g KH$_2$PO$_4$, add 800ml double-distilled H$_2$O, pH with HCl to 7.4 and make up to one litre with double-distilled H$_2$O).

- The slides were then post-fixed by incubation in PBS + 50mM MgCl$_2$ + 4% paraformaldehyde for ten minutes at room temperature, followed by washing in PBS, followed by dehydration through an alcohol series and finally, air-drying.

- Prior to hybridization, 100µl of 70% formamide in 2xSSC was placed on each of the slides under a coverslip and the DNA denatured in an oven at 70°C for five minutes. The coverslips were then removed and the slides plunged into ice-cold 70% ethanol twice, then into 90 and 100% ethanol baths, before being air-dried.

- The YAC DNA mix was then denatured at 75°C for five minutes and preannealed to the metaphase chromosomes for 30 minutes at 37°C.

- The hybridization mix was then placed onto the slides under a coverslip and the coverslip sealed with a rubber solution.

- Hybridization was allowed to proceed in a moist chamber for six days at 37°C.

- After hybridization, the coverslips were removed and the slides washed three times with 50% formamide in 2xSSC for five minutes at 42°C. Further washes at 42°C were performed with just 2xSSC (twice for 2.5 minutes) and 0.1xSSC (twice for 2.5 minutes).

- For signal detection, two preincubation blocking steps were performed, the first for five minutes in 4xSSC, 0.05% Tween 20 detergent and the second for ten minutes in 4xSSC, 5% nonfat dried milk (Marvel). Biotin signal detection was facilitated by a layer of avidin-fluorescein isothiocyanate (FITC) conjugate and biotin-anti-avidin D conjugate (Vector Laboratories, USA), followed by one amplification of signal with avidin-FITC. In each of these cases, conjugate was applied at a concentration of 5µg/ml (in 5% Marvel, 4xSSC) and 100µl/slide for...
twenty minutes under a coverslip. Conjugates were washed off at each stage with 0.05% Tween 20 in 4xSSC for a total of fifteen minutes. Slides were finally washed in 0.9% saline.

• Chromosomes were identified by banding made visible by mounting in antifade medium containing the fluorescent counterstains propidium iodide and DAPI (Vectashield, Vector Laboratories) (antifade medium: 1mg/ml p-phenylenediamine dihydrochloride in 90% glycerol/10% PBS, pH 8.0, plus 0.15µg/ml DAPI and 1µg/ml propidium iodide).

• Viewing of the slides took place on a Nikon Optiphot fluorescent microscope. Images were captured on an MRC 600 Confocal laser scanning microscope or were analyzed using a cooled CCD camera (Photometrics) attached to a Zeiss Axiophot fluorescence microscope equipped with appropriate filters and using Smartcapture software (Digital Scientific).

2.4 Chapter 6: Pulsed field gel electrophoresis of YAC clones bearing human FMO sequences

2.4i Preparation of intact yeast chromosomal DNA for separation by pulsed field gel electrophoresis

Prior to pulsed field gel electrophoresis (PFGE), yeast chromosomal DNA, including YACs was prepared within agarose blocks: A standard genomic DNA preparation would have resulted in excessive breakage of the chromosomes, which needed to be maintained in an intact state. Preparation of yeast chromosomal DNA embedded in agarose blocks was undertaken in the following fashion:

• YAC clones taken from glycerol stocks (Section 2.2, Subsection 2.2iii: Preliminary characterization of requested YAC clones) were used to inoculate 100ml of AHC medium (one litre of AHC medium is made as follows: 1.7g yeast nitrogen base without amino acids and (NH₄)₂SO₄ [Difco], 5g (NH₄)₂SO₄ [Sigma], 10g acid hydrolyzed casein (casamino acids) [Sigma], 20mg adenine hemisulphate [Sigma], add H₂O to one litre and adjust pH to 5.8. After autoclaving, add 50ml filter-sterilized 40% (w/v) glucose and ampicillin [50µg/ml]). Cultures were left to grow for two days with vigorous shaking at 30°C.
• The overnight culture was split into two 50ml tubes (Falcon 2070) and the cells harvested by centrifugation at 1800 rpm (MSE Centaur 2) for five minutes.

• The supernatant was poured off and the cells resuspended in 30ml of 50mM EDTA (pH 8.0).

• The cells were pelleted again exactly as before and this time resuspended in 20ml 50mM EDTA (pH8.0). The resuspended cells of one of the tubes was poured into the other to give 40ml total of resuspended cells in EDTA.

• 10μl of the cell suspension was diluted with 990μl water and the optical density (OD) measured at wavelength 600nm in a spectrophotometer. If the OD reading was higher than 0.5, then the cell suspension was diluted appropriately to achieve a revised OD of between 0.05 and 0.5. The total number of cells in the suspension could be estimated on the basis that one OD unit is equivalent to approximately 2x10⁷ cells.

• Centrifugation was performed once more as before. Cells were resuspended in SCE solution (one litre of SCE: 100ml of 1M trisodium citrate pH 5.8, 120ml of 0.5M EDTA pH 8.0, 182g D-sorbitol, double-distilled H₂O to one litre, sterilization by filtration) to achieve a cell concentration of 2x10⁹/ml.

• A volume of 1M dithiothreitol (DTT) was added to the cell suspension to achieve a final DTT concentration of 70mM.

• Lyticase enzyme (Sigma) was then added at 280 units enzyme per ml of cell suspension. Incubation proceeded at 37°C for five minutes with occasional gentle shaking of the tube. At this point, most of the yeast cells should be spheroplasts.

• An equal volume of 1% low melting point agarose (Sigma), prepared in SCE solution and maintained at 50°C in a waterbath, was added to the spheroplast suspension. The tube was inverted several times to ensure thorough mixing of the agarose and spheroplasts.

• The agarose-spheroplast mixture was immediately placed in the 50°C waterbath. Small volumes of the mixture were then taken from the tube while still in the waterbath and carefully pipetted into alcohol-rinsed disposable plug molds (BIORAD).
• The plug molds were chilled at 4°C for twenty minutes. The solidified agarose blocks were then removed from the molds and placed in 25ml of SCE solution containing 280 units/ml lyticase enzyme. Incubation was allowed to proceed at 37°C for three hours in a waterbath with continual, gentle shaking.

• Following incubation, the SCE-lyticase solution was removed and the blocks immersed in 40ml of lysis solution (100ml lysis solution: 90ml 0.5M EDTA pH 8.0, 1ml 1M Tris-HCl pH 8.0, 1g N-lauroylsarcosine [Sigma], double-distilled H$_2$O to 100ml; just before use, proteinase K [Boehringer Mannheim] from a 10mg/ml stock solution was added to achieve a final concentration of 1mg/ml). Blocks were left to incubate in the lysis solution overnight at 50°C.

• The following day, blocks were allowed to cool at room temperature before removing the lysis solution and replacing it with 40ml of fresh lysis solution. Incubation was allowed to proceed at 50°C for a further four hours.

• After incubation, the blocks were once more allowed to cool at room temperature, before replacing the lysis solution with 30ml of 50mM EDTA pH 8.0. The tube containing the blocks and EDTA solution was gently shaken by placing on a rocking platform in a 4°C cold room. Over the following 24 hours, the EDTA solution was replaced five times while maintaining gentle agitation and the 4°C temperature.

At this point, the agarose blocks were assumed to contain intact yeast chromosomal DNA, with very little else in the way of other macromolecules and cellular debris. As such, the blocks could be stored at 4°C in 50mM EDTA almost indefinitely until required for use.

2.4ii Pulsed field gel electrophoresis
The equipment used to perform the pulsed field gel electrophoresis (PFGE) was a BIORAD CHEF-DRII Drive Module with accompanying BIORAD Pulsewave 760 Electrophoretic Field Switcher. PFGE protocol was as follows;

• 1.5% agarose for pulsed field (Sigma) was melted in 0.5xTBE buffer (1x TBE: 90mM Tris-HCl, 90mM boric acid, 2mM EDTA, pH 8). Agarose was allowed to cool to 50°C by placing in a waterbath at this temperature.

• The gel casting mold and comb (twenty teeth) were cleaned thoroughly with ethanol.
The previously-prepared agarose blocks containing yeast chromosomal DNA (see above) were cut into three evenly sized pieces with a scalpel. The pieces were then equilibrated in 0.5xTBE buffer with two fifteen minute washes in 7ml Sterilin tubes.

Using a spatula, the agarose pieces were transferred to the teeth of the comb by placing the bottom edge of each block even with the bottom edge of each tooth. The agarose pieces adhered to the comb teeth by virtue of surface tension. The comb was then placed in the gel chamber with the agarose pieces facing the anode.

The agarose was very carefully poured into the gel chamber, taking care not to dislodge the agarose pieces from the comb. Gel solidification was allowed to proceed at room temperature. After setting of the gel, the comb was carefully removed, leaving the agarose pieces behind in the gel.

Marker DNA was inserted into the spare wells (i.e. those wells which did not have an adjacent agarose piece). Two markers were used: λ Pulse Marker, consisting of 21 λ concatemers of molecular weights approximately 48.5-1019kb (Sigma), and intact yeast chromosomes of Saccharomyces Cerevisiae strain YPH80 (Sigma) (sixteen chromosomes in total ranging in size from 225kb to 2200kb). Both markers are provided in 1% low melting point agarose at a concentration of 1µg DNA per 20µl agarose. Slices of just the right size for insertion into the wells of the gel were cut with a scalpel.

The gel was placed in the precooled (4°C) electrophoresis apparatus. 0.5xTBE (also precooled to 4°C) was poured until the gel was completely submerged. Electrophoresis was performed for eighteen hours, using 5V/cm electric field strength and a one minute pulse time. During electrophoresis, the apparatus was maintained at a constant 4°C.

After completion of the run, the gel was carefully removed and stained by placing in a solution containing 1ng/ml ethidium bromide (BDH Merck). DNA bands were viewed on a UV transilluminator.

2.4iii Southern hybridization of pulsed field gel
The protocols for Southern blotting, nylon membrane prehybridization and hybridization, FMO cDNA probe radiolabelling, nylon membrane washing and autoradiography were all exactly the same as described above for Section 2.2:
Chapter 4: Isolation of YACs bearing FMO-containing human genomic inserts.

2.5 Chapter 7: Screening of a human chromosome 1-specific cosmid library for FMO genes

2.5i ICRF human chromosome 1-specific cosmid library
The human chromosome 1-specific cosmid library (library No. 112 [L4/FS1]) was provided by the ICRF Reference Library Database (RLDB). The library was constructed by Dean Nizetic from digests of DNA from '4x cell line (LCL 127)' and was ligated into the cosmid vector Lawrist 4. Specifically, Lawrist 4 was cut with the restriction enzyme BamH1, whereas the genomic DNA was partially cut with Mbol. Upon ligation of these two respective sticky ends, only an average of 25% of sites will be restored to a sequence recognized by BamH1. As host, DH5 alpha MCR (BRL) was used.

The entire library exists as a single nylon filter on which has been spotted 48x48 blocks of cosmid clones in an ordered grid formation, giving 18,432 cosmid clones in total. The actual library consists of just 9,216 individual cosmid clones, but these have been spotted in duplicate on the nylon filter.

2.5ii Library screening
Prehybridization and hybridization of the library nylon filter were undertaken in a manner analogous to that described for prehybridization and hybridization of Southern blots in Section 2.2, Subsections 2.2i (Prehybridization of YAC library nylon filters) and 2.2v (Probe hybridization to YAC library nylon filters), respectively. Also, the generation of the full-length FMO cDNA probes and their radiolabelling was performed in the same manner as is described in Section 2.2, Subsections 2.2iii (Preparation of FMO cDNA probes) and 2.2iv (Radiolabelling of FMO cDNAs). Lawrist 8 cosmid vector radiolabelling was undertaken by performing the following reaction:

- 2.5µl Lawrist 8 vector (approximately 100ng DNA)*
- 6µl 20mM dithiothreitol (DTT) (Sigma)
- 4µl [α³⁵S]-dATP, 800 Ci/mmol & 10µCi/µl (NEN-Dupont)
• 1.5μl dNTP mix: 5mM concentration of dTTP, dCTP & dGTP, respectively (Pharmacia)

• 6μl 10x Random primer (RP) buffer (900mM HEPES adjusted to pH6.6 with 4M NaOH, 100mM MgCl₂)

• 1.5μl Random hexadeoxyribonucleotide primers (Sigma), previously diluted in TE buffer to 90 OD units/ml*

• Double-distilled H₂O to a final volume of 60μl*

• 1μl (10 units) Klenow fragment of *E.coli* DNA polymerase I (Pharmacia)**

*Lawrist DNA, random primers and H₂O were boiled separately together for five minutes followed by chilling on ice, before adding the DTT, dNTPs, [α³⁵S]-dATP and RP buffer.

**Klenow polymerase was added last of all.

After mixing the constituents of the reaction, incubation proceeded for a minimum of one hour at 37°C, or three hours if left at room temperature.

Removal of unincorporated nucleotides and measurement of probe specific activity was performed in the same manner as described for the *FMO* cDNA probes. Radiolabelled Lawrist 8 was added to the hybridization medium at a final concentration of approximately 7ng/ml.

The hybridized nylon filter was washed as follows;

• 30 minutes at 65°C in 0.1xSSPE (20xSSPE: 3M NaCl, 0.02M EDTA, 0.2M Na₂HPO₄), 1% sodium dodecyl sulphate (SDS)

• Repeat

The washed filter was wrapped in Saran Wrap and autoradiography performed overnight initially. Positive clones were identified by examination of the X-ray film and their coordinates established accordingly. Upon submission of the coordinates of the positive clones to the RLDB, they were dispatched as stabs on agar.
2.5iii Verification of requested clones (replica plating)

Upon receipt of the cosmid clones, each was inoculated into 10ml of LB broth (BIO101 LB medium capsules) with 20μg/ml kanamycin antibiotic (Sigma) in 30ml Sterilin Universal tubes. Kanamycin resistance is conferred by the Lawrist 4 cosmid vector. Cells were allowed to grow overnight by vigorous shaking in a waterbath at 37°C.

LB agar plates (140mm) containing 20μg/ml kanamycin were prepared. 100μl of a 1/1000 dilution of the overnight cell cultures were spread evenly over the plates. Colonies were left to grow by incubating the plates overnight at 37°C.

The following day, Hybond-N+ nylon discs (Amersham) were laid on top of the agar plates. Immediately, a syringe needle was used to puncture the disc and agar in a patterned formation, so as to provide a reference for orienting the exposed X-ray film and agar plate later. The discs were left for approximately one minute before carefully lifting from the plates. The agar plates were returned to a 37°C incubator to allow regrowth of lifted colonies. Exposure of resident nucleic acids within the cells of colonies transferred to the nylon discs, and their subsequent covalent attachment to the nylon discs, were achieved as follows;

- Lifted nylon discs were placed on Whatmann 3MM paper soaked in denaturing solution (denaturing solution: 1.5M NaCl, 0.5M NaOH), with colonies facing upwards, for seven minutes.

- Discs were then transferred to 3MM paper soaked in neutralizing solution (neutralizing solution: 1.5M NaCl, 0.5M Tris-HCl pH7.4, 1μM EDTA), colony side up, for three minutes.

- The previous neutralization step was repeated.

- Discs were quickly rinsed in 2xSSPE.

- Cross-linking of the DNA was ensured by placing the discs, DNA side facing upwards, on 3MM paper soaked in 20xSSPE, and selecting the Auto Cross Link function (120 mjoules) of a UV Stratalinker (Stratagene).

- Discs were dried in an 80°C oven and stored wrapped in Saran Wrap.

The subsequent prehybridization and hybridization of the colony lift nylon discs, FMO cDNA probe radiolabelling, washing and autoradiography were all
performed in the manner described in Section 2.2 (Subsections 2.2ii, 2.2v, 2.2iv, 2.2vi and 2.2vii, respectively).

Exposed X-ray films resulting from autoradiography of the \textit{FMO} cDNA-probed nylon discs were used to go back to the agar plates and identify those colonies that appeared to have strongly hybridized to the probes. Such colonies were picked and used to inoculate 10ml of LB medium with 20\(\mu\)g/ml kanamycin in 30ml Sterilin Universal tubes. Growth was allowed to proceed overnight at 37°C with vigorous shaking.

The following day, glycerol stocks were made for each of the colonies grown by taking 150\(\mu\)l of the respective cultures and adding to 850\(\mu\)l of glycerol in a sterile Eppendorf tube. Tubes were snap frozen in liquid nitrogen and stored at -70°C.

2.5iv Southern blotting studies
Glycerol stocks were used to inoculate conical flasks containing 100ml LB medium with 20\(\mu\)g/ml kanamycin. Cells were allowed to grow overnight by shaking vigorously at 37°C. The following day, cells were harvested in 250ml bottles by centrifugation at 4 000 rpm for ten minutes at 4°C in an MSE 18 centrifuge.

Preparation of cosmids DNA was achieved by the use of Qiagen Plasmid Maxi Kits. DNA pellets at the end of the preparation were resuspended in 200\(\mu\)l of TE buffer (10mM Tris-HCl, 1mM EDTA, pH7.4). DNA yield was estimated by measuring optical density at wavelength 260nm (multiplication by the dilution factor and 50 gave DNA concentration as \(\mu\)g/ml).

Cosmid preparations of the respective clones to be analyzed by Southern hybridization were digested with the restriction endonuclease BamH1 in the following manner;

• 5\(\mu\)g cosmid DNA (typically 5-40\(\mu\)l of Qiagen preparation)

• 20 units BamH1 (Pharmacia)*

• 11\(\mu\)l 10x One-Phor-All buffer (Pharmacia)

• Double-distilled \(\text{H}_2\text{O}\) to 55\(\mu\)l
Incubation was allowed to proceed at 37°C for two hours with just 10 units of BamH1, following which a further 10 units were added and incubation continued for a further two hours.

Digests were checked by running 5μl of the reactions, plus 0.5μl 10x loading dye (10ml of 10x loading dye: 25mg bromophenol blue, 4ml glycerol, 6ml H2O) on a 0.7% agarose (Sigma) gel made with 1xTBE buffer (1xTBE: 90mM Tris-HCl, 90mM boric acid, 2mM EDTA, pH 8) and 0.5ng/ml ethidium bromide (BDH Merck).

The remainder of the reactions were used for Southern blotting. Samples plus loading dye were loaded on a 0.7% agarose-TBE gel with 0.5ng/ml ethidium bromide. Also loaded were radiolabelled (35S) molecular weight standards (1kb ladder: Gibco-BRL Life Technologies), which were prepared in the manner described above in Section 2.2 Subsection 2.2viii (Preliminary characterization of requested YAC clones).

Following separation, Southern blotting proceeded exactly as described in Section 2.2, Subsection 2.2viii. The methods used to probe these blots with FMO cDNAs were also the same as described in Section 2.2.

2.6 Chapter 8: Analysis of cosmid clones isolated from a chromosome 1-specific library by fluorescence in-situ hybridization to human metaphase chromosomes

Cosmid DNA was prepared in the manner described in Section 2.5, Subsection 2.5iv (Southern blotting studies). The same protocols were employed in using these cosmid clones for fluorescence in-situ hybridization to human metaphase chromosomes as were described for YACs in Section 2.3 (Chapter 5: Fluorescence in-situ hybridization analysis of YAC clones bearing human genomic sequences containing FMO genes).
Chapter 3

Localization of human \textit{FMO2} and \textit{FMO5} genes to chromosome 1q
3.1 Background

Research has already been published concerning the chromosomal localization of human \textit{FMO1} (104), \textit{FMO3} (171) and \textit{FMO4} (84). (Note: at the time that \textit{FMO4} was localized it was labelled \textit{FMO2}. Subsequently, the nomenclature system has been rationalized (31).) All three genes were shown to be localized to chromosome 1q, raising the possibility that the entire human \textit{FMO} gene family exists in a clustered formation. In each of these cases, this was established by using the polymerase chain reaction (PCR) to screen a range of human-rodent somatic cell hybrids. These hybrids contain one or more human chromosomes (or even just the long or short arm of a particular chromosome) of known identity. Availability of entire cDNA sequences for the three genes (84, 104, 132) enabled the design of primers for the PCR reactions that amplified entirely from within the 3' non-coding region. It was shown that the generation of amplification products using these primers was a specific indicator for the presence of the gene concerned. By using these primers to screen a panel of hybrids bearing a sufficient variation in human chromosome content, it was possible to logically deduce first, on which human chromosome the \textit{FMO} gene was located and secondly, refine the location to either the long (q) or short (p) arm of that chromosome.

In the case of \textit{FMO1}, the localization of the gene has been taken further (171) through the use of an \textit{in-situ} hybridization technique (343). Human metaphase chromosomes spreads obtained from lymphocyte cultures were banded using Giemsa stain (G-banded) and hybridized to radiolabelled \textit{FMO1} cDNA. Autoradiography showed the probe to have hybridized at 1q23-25.

It was considered of some interest whether the two known remaining members of the human \textit{FMO} gene family yet to be mapped to a chromosome, \textit{FMO2} and \textit{FMO5}, also reside on chromosome 1q, thereby furthering the possibility of a gene cluster in this region for the entire family. This was investigated using similar techniques to those employed for the \textit{FMO1}, \textit{FMO3}, and \textit{FMO4} localization studies (172).

3.2 Chromosomal localization of the human \textit{FMO2} gene

PCR reactions were carried out using primers designed (from the available cDNA sequence) to anneal to sequences located on either side of the translation termination codon. Amplification with these primers yielded a fragment 252bp
in size when using human genomic DNA or complete \textit{FMO2} cDNA as template, the majority of which was derived from the 3’ untranslated region of the mRNA. Production of this fragment was shown to be a specific indicator for the presence of the human \textit{FMO2} gene. PCR reaction conditions and primer descriptions are listed in Chapter 2 (Materials and Methods), Section 2.1. Amplification products generated in this manner were analyzed by 2% agarose gel electrophoresis.

Genomic DNA preparations, isolated from a panel totaling twelve human-rodent somatic cell hybrids, were used as templates in these reactions. The names of these hybrids (and three others used in later work) are listed in Table 3.1, along with their respective genomic components. The twelve hybrids were selected to provide unequivocal assignment of gene location based on the presence or otherwise of the 252bp amplification product specific for the presence of \textit{FMO2}. Figure 3.1 shows an agarose gel on which were separated PCR reactions for ten of the twelve hybrids. The reactions for the other two hybrids were run at a later date on a separate gel and this is shown in Figure 3.2. Table 3.2 lists the karyotype of all hybrids screened and states whether they directed the amplification of the \textit{FMO2}-specific fragment, based on examination of these two gels.

\textit{FMO2} primers are shown to direct the amplification from human genomic DNA of a DNA fragment of the expected size, i.e. 252bp (Figure 3.1, Track c and Figure 3.2, Track c: relative positions marked by an arrow). Such a fragment is absent when no DNA (Figure 3.1, Track b and Figure 3.2, Track b), mouse genomic DNA (Figure 3.1, Track d and Figure 3.2, Track d) or hamster genomic DNA (Figure 3.1, Track e and Figure 3.2, Track e) is included, confirming it to be an indicator of the presence of the human \textit{FMO2} gene alone (each of the hybrid clones was either a mouse-human or hamster-human hybrid, see Table 3.1). \textbf{Note}: In both Figures 3.1 and 3.2, amplification products of varying sizes other than the 252bp \textit{FMO2}-specific fragment can be seen in the form of additional bands in the gel for some of the reactions. However, where such amplification products are seen to have been produced using the hybrid DNA as templates, a corresponding band can be seen to exist for the reaction in which the same rodent genome as was present in that hybrid has been used as the sole template. For example, examination of Figure 3.1 reveals that two regions of the mouse genome were amplified in significant amounts, producing clearly visible bands in the gel of approximate size 180bp and 380 bp when mouse genomic DNA alone was the template (Figure 3.1, Track d).
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<th>Hybrid Name</th>
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Table 3.1. Name and nature of somatic cell hybrids used for human *FMO2* and *FMO5* localization studies. Hybrids 1-12 are those that were used to determine chromosomal localization of *FMO2* and *FMO5*. Hybrids 13-15 were used for the regional localization studies.
Figure 3.1. PCR-screening of ten human-rodent somatic cell hybrids for the presence of the FMO2 gene. Shown here are DNA products generated by PCR amplification with primers specific for the FMO2 gene, made visible after electrophoretic separation through a 2% agarose gel. DNA template samples were from human liver (c), the mouse cell line RAG (d), the hamster cell line a23 (e), or the somatic cell hybrids CTP34B4 (f), MOG34A4 (g), EDag3R (h), Twin19D12 (i), Twin19F9 (j), DUR4.3 (k), DUR4R3 (l), Twin19C5 (m), GM10114 (n) and Twin19F6 (o). No-DNA control (b). Molecular weight standards (Gibco-BRL 1-kb ladder) (a and p). Note: the FMO2-specific fragment is approximately 250bp in size. An arrow marks the approximate position it can be found to have travelled through the gel.
Figure 3.2. PCR-screening of two further human-rodent somatic cell hybrids for the presence of the \textit{FMO2} gene. Shown here are DNA products, generated by PCR amplification with primers specific for the \textit{FMO2} gene, made visible after electrophoretic separation through a 2\% agarose gel. DNA template samples were as follows: human genomic DNA (lane c), mouse genomic DNA (lane d), hamster genomic DNA (lane e), human-hamster somatic cell hybrid GM07299 (lane f) and human-mouse somatic cell hybrid 2860H7 (lane g). No-DNA control is in lane b. Marker DNA fragments (Gibco-BRL 1-kb ladder) are loaded in lanes a and h. \textit{Note}: the \textit{FMO2}-specific fragment is approximately 250bp in size. An arrow marks the approximate position it can be found to have travelled through the gel.
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</table>

Chromosome/
FMO concordance
++          | 6 | 2 | 5 | 3 | 3 | 4 | 2 | 3 | 1 | 2 | 0 | 5 | 2 | 5 | 0 | 3 | 4 | 5 | 2 | 2 | 4 | 3 | 2 |
--          | 6 | 5 | 2 | 4 | 3 | 5 | 6 | 3 | 6 | 5 | 4 | 2 | 2 | 2 | 5 | 5 | 2 | 2 | 6 | 2 | 2 | 2 | 5 |

Discordance
+-          | 6 | 4 | 1 | 3 | 3 | 1 | 4 | 3 | 5 | 4 | 6 | 1 | 3 | 1 | 5 | 2 | 2 | 1 | 4 | 4 | 2 | 3 | 3 |
-+          | 6 | 1 | 4 | 2 | 3 | 1 | 0 | 3 | 0 | 1 | 2 | 4 | 2 | 4 | 0 | 1 | 4 | 4 | 0 | 4 | 4 | 4 | 0 |

Table 3.2. Screening of twelve human-rodent somatic cell hybrids by PCR for the presence of the human FMO2 gene to determine chromosome location. The data indicates which chromosome(s), in whole or part, are present within each of twelve hybrids and whether they were capable of directing the amplification of a PCR amplification product specific for the presence of human FMO2. Concordance and discordance figures are also shown for each chromosome. *Note:* +, human chromosome present; -, human chromosome not detected; 0, only part of chromosome present.
Both of these bands can also be seen for the human-mouse hybrids CTP34B4 (Figure 3.1, Track f), MOG34A4 (Figure 3.1, Track g), EDag3R (Figure 3.1, Track h), DUR4.3 (Figure 3.1, Track k) and DUR4R3 (Figure 3.1, Track l). Hence, the primers used in these reactions are clearly capable of annealing to regions of the hamster and mouse genomes under the conditions used, while remaining valid for determining the presence of human FM02.

Hybrids not containing human chromosome 1, i.e. DUR4.3 (Figure 3.1, Track k), DUR4R3 (Figure 3.1, Track l), Twin19C5 (Figure 3.1, Track m), GM10114 (Figure 3.1, Track n), Twin 19F6 (Figure 3.1, Track o) and 2860H7 (Figure 3.2, Track g) were unable to direct amplification of the FM02-specific fragment.

Only hybrids containing human chromosome 1, i.e. CTP34B4 (Figure 3.1, Track f), MOG34A4 (Figure 3.1, Track g), EDag3R (Figure 3.1, Track h), Twin19D12 (Figure 3.1, Track i), Twin 19F9 (Figure 3.1, Track j) and GM07299 (Figure 3.2, Track f) were able to direct amplification of the FM02-specific fragment.

From this, there can be little doubt that, as with human FMO1, FMO3 and FMO4, human FM02 resides on chromosome 1.

3.3 Chromosomal localization of the human FMO5 gene

Identifying the chromosomal location of the FMO5 gene involved using a protocol very similar that used for localizing the FMO2 gene. PCR reactions were carried out using primers designed (from the cDNA sequence) to prime amplification of a DNA fragment 403bp in size, the sequence of which is located entirely within the 3' untranslated region of the mRNA. It had previously been shown that the amplification of this fragment was a specific indicator for the presence of FMO5. Once again, PCR reaction conditions and primer descriptions are listed in Chapter 2 (Materials and Methods), Section 2.1, and amplification products generated in this manner were analyzed by 2% agarose gel electrophoresis.

Genomic DNA isolated from the same twelve human-rodent somatic cell hybrids as were used for FMO2 localization were used as templates (Table 3.1). Hence, the panel was intended to demonstrate unequivocal assignment of FMO5 chromosome location based on the presence or otherwise of the gene-specific amplification product. Figure 3.3 shows an agarose gel on which the PCR reactions for eight of these hybrids were separated (two are not included,
MOG34A4 and Twin19D12, although the reactions were performed and their outcome is listed in Table 3.3). Figure 3.4 shows another gel on which the PCR reactions of two further hybrids were separated. Table 3.3 lists all twelve hybrids screened, their karyotype and whether they were able to direct amplification of the \textit{FM05}-specific fragment.

\textit{FM05} primers are shown to direct the amplification from human genomic DNA of a fragment of the expected size i.e. 403bp (Figure 3.3, Track c and Figure 3.4, Track c [untypically faint on this occasion]). An arrow marks the approximate positions on Figures 3.3 and 3.4 where this fragment has migrated to. Such a fragment is absent when no DNA (Figure 3.3, Track b and Figure 3.4, Track b), rat genomic DNA (Figure 3.3, Track d and Figure 3.4, Track d) or hamster genomic DNA (Figure 3.3, Track e and Figure 3.4, Track e) is included, confirming it to be an indicator of the presence of the human \textit{FM05} gene alone. As was the case for the \textit{FM02} primers, however, the primers used here are seen to be capable of annealing to regions of the mouse and hamster genomes under the reaction conditions used. Hence, amplification products can be seen apart from the 403bp \textit{FM05}-specific fragment. For example, examination of Figure 3.3 reveals that when mouse genomic DNA is the only template added, a very clear band of approximate size 850bp appears in the gel (Track d). This band can also be seen for the human-mouse hybrids CTP34B4 (Track f), EDag3R (Track g) and DUR4R3 (Track j). On the other hand, Figure 3.3 shows that hamster genomic DNA alone most clearly amplifies a fragment of approximate size 500bp (Track e). The corresponding band in the gel can also be seen for the human-hamster hybrid Twin19F9 (Track h). The additional bands produced using these primers, however, does not affect their ability to accurately indicate the presence or otherwise of the \textit{FM05} gene.

Human-rodent somatic cell hybrid clones that do not contain human chromosome 1, i.e. DUR4.3 (Figure 3.3, Track i), DUR4R3 (Figure 3.3, Track j), Twin19C5 (Figure 3.3, Track k), GM10114 (Figure 3.3, Track l), Twin19F6 (Figure 3.3, Track m) and 2860H7 (Figure 3.4, Track g), also fail to produce the \textit{FM05}-specific fragment. Only hybrids containing human chromosome 1, i.e. CTP34B4 (Figure 3.3, Track f), EDag3R (Figure 3.3, Track g), Twin19F9 (Figure 3.3, Track h), GM07299 (Figure 3.4, Track f), MOG34A4 (not shown) and Twin19D12 (not shown) are able to direct amplification of the \textit{FM05}-specific fragment.

From this, it can be concluded that the human \textit{FM05} gene, along with \textit{FM01}, \textit{FM02}, \textit{FM03}, and \textit{FM04}, resides on chromosome 1.
Figure 3.3. PCR-screening of ten human-rodent somatic cell hybrids for the presence of the *FMO5* gene. Shown here are DNA products generated by PCR amplification with primers specific for the *FMO5* gene, made visible after electrophoretic separation through a 2% agarose gel. DNA template samples were from human liver (c), the mouse cell line RAG (d), the hamster cell line a23 (e), or from the somatic cell hybrids CTP34B4 (f), EDAg3R (g), Twin19F9 (h), DUR4.3 (i), DUR4R3 (j), Twin19C5 (k), GM10114 (l) and Twin19F6 (m). No-DNA control (b). Molecular weight standards (Gibco-BRL 1-kb ladder) are shown in lanes a and n. Note: the *FMO5*-specific fragment is approximately 400bp in size. An arrow marks the approximate position it can be found to have travelled through the gel.
Figure 3.4. PCR-screening of two further human-rodent somatic cell hybrids for the presence of the *FMO5* gene. Shown here are DNA products, generated by PCR amplification with primers specific for the *FMO5* gene, made visible after electrophoretic separation through a 2% agarose gel. DNA template samples were as follows: human genomic DNA (lane c), mouse genomic DNA (lane d), hamster genomic DNA (lane e), human-hamster somatic cell hybrid GM07299 (lane f) and human-mouse somatic cell hybrid 2860H7 (lane g). No-DNA control is in lane b. Marker DNA fragments (Gibco-BRL 1-kb ladder) are loaded in lanes a and h. *Note*: the *FMO5*-specific fragment is approximately 400bp in size. An arrow marks the approximate position it can be found to have travelled through the gel.
Table 3.3. Screening of twelve human-rodent somatic cell hybrids by PCR for the presence of the human *FMO5* gene to determine chromosome location. The data indicates which chromosome(s), in whole or part, are present within each of twelve hybrids and whether they were capable of directing the amplification of a PCR amplification product specific for the presence of human *FMO5*. Concordance and discordance figures are also shown for each chromosome. Note: +, human chromosome present; -, human chromosome not detected; 0, only part of chromosome present.

| Hybrid          | Reference | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | X  |
|-----------------|-----------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|---|---|
| Twin19F9        | 349       | + | + | + | + | - | - | + | - | + | - | + | + | + | + | + | + | + | + | - | + | + | + | + |   |
| MOG34A4         | 350       | + | - | + | + | + | - | - | + | - | + | + | - | + | + | - | + | + | + | + | + | + | + | + |   |
| CTP34B4         | 351       | + | + | - | + | + | + | - | + | - | - | + | + | - | + | + | + | + | + | + | + | + | + | + |   |
| EDeq3R          | 352       | + | - | - | - | + | 0 | + | - | - | + | - | + | + | 0 | 0 | + | + | + | + | - | + | + | + | + |   |
| Twin19D12       | 349       | + | - | + | - | + | - | + | - | + | - | - | + | + | + | + | - | + | + | - | + | + | + | + |   |
| GM07299         | 353       | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |   |
| Twin19F6        | 349       | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |   |
| DUR4.3          | 349       | + | + | + | - | + | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |   |
| DUR4R3          | 354       | - | - | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |   |
| Twin19C5        | 349       | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |   |
| GM10114         | 355       | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |   |
| 2860H7          | 353       | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |   |

Chromosome/ FMO concordance

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Discordance

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</tr>
<tr>
<td>-+</td>
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Note: +, human chromosome present; -, human chromosome not detected; 0, only part of chromosome present.
3.4 Further localization studies

I have shown that the human *FMO2* and *FMO5* genes reside on chromosome 1. Human-rodent somatic cell hybrids are available with only the long (q) or short (p) arms of human chromosomes, thereby allowing genes to be further localized. In the case of the human *FMO1* (17q), *FMO2* (17q) and *FMO3* (17q) genes, once they had been shown to reside on chromosome 1 they were further localized to 1q by PCR-screening of hybrids containing either 1p or 1q. The same principles were used to determine whether the genes for human *FMO2* and *FMO5* also reside on 1q.

3.4i Regional localization of the human *FMO2* gene

Three human-rodent somatic cell hybrids were screened by PCR using identical primers to those used for localizing human *FMO2* to chromosome 1 (see Section 3.2: *Chromosomal localization of the human FMO2 gene*, above). Performing PCR with these primers generates a 252bp DNA fragment only when the *FMO2* gene is present to act as template. Two of the hybrids (CON5E and F4sc13cL12) contained human 1p and the other (CON2) contained human 1q. Which of these hybrids were able to direct amplification of the *FMO2*-specific DNA fragment would determine on which arm of chromosome 1 human *FMO2* resides.

Figure 3.5 shows an agarose gel on which these reactions were separated. The hybrid CON2, which contains human chromosome 1q, was able to generate the *FMO2*-specific amplification product (Figure 3.5, Track d), in common with human genomic DNA (Figure 3.5, Track c). Hybrids CON5E (Figure 3.5, Track e) and F4sc13cL12 (Figure 3.5, Track f), which contain human chromosome 1p, failed to do so, as did the no-DNA control (Figure 3.5, Track b). Note that mouse genomic DNA only and hamster genomic DNA only control reactions were not run on the gel, even though the rodent component of these three hybrids was of one of these two genomes. This was because PCR reactions in the presence of these genomes alone had already been performed using identical primers, (see Section 3.2: *Chromosomal localization of the human FMO2 gene*, above), when it was shown that the *FMO2*-specific amplification product was not generated in either case (Figure 3.1, Tracks d and e, respectively).

From this data we conclude that the human *FMO2* gene resides on chromosome 1q (Table 3.4), along with the *FMO1*, *FMO3* and *FMO4* genes.
Figure 3.5. PCR-screening of three human-rodent somatic cell hybrids for the presence of the *FMO2* gene. Shown here are DNA products generated by PCR amplification with primers specific for the *FMO2* gene, made visible after electrophoretic separation through a 2% agarose gel. DNA template samples were from human liver (c) and the human-rodent somatic cell hybrids CON2 (d), CON5E (e) and F4sc13cL12 (f). No-DNA control (b). Molecular weight standards (Gibco-BRL 1-kb ladder) (a and g). The size of the *FMO2*-specific amplification product is approximately 250bp and is identified by the arrow.
Table 3.4. Regional localization of the human *FMO2* gene by PCR analysis of human-rodent somatic cell hybrids. Three hybrids were screened with primers capable of amplifying a DNA product only in the presence of the human *FMO2* gene. Two of the hybrids, CON5E and F4sc13cL12, bear only the short arm (p) of human chromosome 1 and these failed to produce the *FMO2*-specific amplification product. However, the other clone, CON2, which bears the long arm of human chromosome 1 (q), did direct amplification of the *FMO2*-specific fragment. From this, we conclude that the human *FMO2* gene resides on chromosome 1q.

<table>
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<th>Arm of human chromosome 1 present in hybrid</th>
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<td>p</td>
<td>−</td>
</tr>
<tr>
<td>F4sc13cL12</td>
<td>349</td>
<td>p</td>
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3.4ii Regional localization of the human *FM05* gene

The same three hybrids as were used in the regional localization of human *FMO2*, CON2 (bearer of human chromosome 1q), CON5E (bearer of human chromosome 1p) and F4sc13cL12 (bearer of human chromosome 1p), were used to determine the regional localization of human *FM05* via PCR. Primers used were the same as those used to assign human *FM05* to chromosome 1 (see Section 3.3: Chromosomal localization of the human *FM05* gene, above). Only when the human *FM05* gene is present will these primers direct amplification of a DNA fragment approximately 400bp in size.

Figure 3.6 shows an agarose gel on which these reactions were separated. Of the three hybrids, only CON2 (Figure 3.6, Track c), which bears human chromosome 1q, was able to direct amplification of the *FM05*-specific amplification product, in common with human genomic DNA (Figure 3.6, Track b). The other two hybrids, CON5E (Figure 3.6, Track d) and F4sc13cL12 (Figure 3.6, Track e), which contain human chromosome 1p, failed to produce this fragment, as did the no-DNA control (Figure 3.6, Track a). Note that mouse genomic DNA only and hamster genomic DNA only control reactions were not run on the gel, even though the rodent component of these three hybrids consisted of one of these two genomes. This was because PCR reactions in the presence of these genomes had already been performed using identical primers (see Section 3.3: Chromosomal localization of the human *FM05* gene, above), when it was shown that the *FM05*-specific amplification product was not generated in either case (Figure 3.3, Tracks d and e, respectively)

We can conclude that the human *FM05* gene resides on chromosome 1q (Table 3.5), as is the case for the human *FMO1, FMO2, FMO3* and *FMO4* genes.

3.5 Implications

As has previously been mentioned, genes coding for *FMO1* (104), *FMO3* (171) and *FMO4* (84) have previously been mapped to human chromosome 1. Furthermore, regional mapping studies have shown each of these genes to be located on the long arm of this chromosome (171), and FMO1 has been further localized to 1q23-q25 (171).

The results shown here demonstrate that the two remaining known genes in the human *FMO* family, *FMO2* and *FM05*, also reside on chromosome 1q. *FMO* genes appear to have arisen from a common ancestral gene, via gene duplication
events, some 250-300 million years ago (84, 171). The presence on chromosome 1q of genes coding for all five known members of the FMO human family suggests that, subsequent to these duplication events, the entire FMO gene family has remained linked, possibly in a single clustered formation.
Figure 3.6. PCR-screening of three human-rodent somatic cell hybrids for the presence of the \textit{FMOS5} gene. Shown here are DNA products generated by PCR amplification with primers specific for the \textit{FMOS5} gene, made visible after electrophoretic separation through a 2% agarose gel. DNA template samples were from human liver (b) and the human-rodent somatic cell hybrids CON2 (c), CON5E (d) and F4sc13c12 (e). No-DNA control (a). Molecular weight standards (Gibco-BRL 1-kb ladder) (f). The human \textit{FMOS5}-specific amplification product is approximately 400bp in size. The position of this fragment on the gel has been marked by an arrow.
Hybrid Reference Arm of human chromosome 1 present in hybrid Human FMO5-specific amplification product

<table>
<thead>
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<th>Hybrid</th>
<th>Reference</th>
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<th>Human FMO5-specific amplification product</th>
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Table 3.5. Regional localization of the human FMO5 gene by PCR analysis of human-rodent somatic cell hybrids. Three hybrids were screened with primers capable of amplifying a DNA product only in the presence of the human FMO5 gene. Two of the hybrids, CON5E and F4sc13cL12, bear only the short arm (p) of human chromosome 1 and these failed to produce the FMO5-specific amplification product. However, the other clone, CON2, which bears the long arm of human chromosome 1 (q), did direct amplification of the FMO5-specific fragment. From this, we conclude that the human FMO5 gene resides on chromosome 1q.
Chapter 4

Isolation of YACs bearing \textit{FMO}-containing human genomic inserts
4.1 Background

All five known human FMO genes have been assigned to chromosome 1q (Chapter 3: Localization of human FMO2 and FMO5 genes to chromosome 1q), raising the possibility that this gene family exists as a cluster. A way to begin exploring this possibility would be to isolate genomic fragments spanning the respective FMO genes. Depending on the size of the isolated fragments, and the relative spacing of the genes along the chromosome, two or more genes might be isolated on one fragment or (via "chromosome walking") further clones isolated to generate a contiguous sequence spanning the cluster, if it exists. Furthermore, isolating such sequences would inevitably yield flanking promoter regions amenable to further study (computerized analysis of elucidated sequence, gel retardation/DNA mobility shift assay, DNase I footprinting assay and so forth). From such data, mechanisms underlying the developmental and tissue-specific patterns of expression witnessed by members of the FMO gene family may be understood. Indeed, if the gene family is clustered, this may form an intrinsic part of the expression regulation mechanism. Finally, it remains a possibility that other, hitherto undiscovered, members of the human FMO gene family exist in addition to the five discovered so far. Indeed, Lawton et al have stated that mixed-probe, low stringency analysis of human genomic DNA indicates that one, and possibly two, more FMO genes remain to be identified (344). There seems a fair chance that isolation of large genomic fragments containing the known FMO genes might also contain these undiscovered gene(s) if they exist, especially as all five members discovered so far are located at a relatively similar genome location and may be clustered.

To date, there have been no reports of the isolation of genomic fragments spanning the entire gene for any of the human FMOs in the published literature or through personal communications. Sequence data from the flanking promoter regions of these genes are also not available at present. Some 5' flanking sequence has recently been obtained in the case of rabbit FMO2, however (182).

Consequently, human genomic libraries constructed using either cosmid or yeast artificial chromosomes (YACs) as vectors were screened for FMO sequences. The former would provide inserts 'only' up to 40kb in length but would be easier to characterize and manipulate than YACs, which would have the advantage of being, on average, 600kb in length and so more likely to span two or more genes if a cluster exists. The screening of cosmid libraries is discussed fully in Chapter 7 (Screening of a human chromosome 1-specific
cosmid library for *FMO* genes). Here, the outcome of screening a YAC human genomic library provided by ICRF and supplied by the UK HGMP Resource Centre is discussed. This library consists of high density colony filters. In all, 20,500 clones comprising the genomes of two females and one male are gridded across fourteen filters (for more technical details regarding this library and all methodological procedures mentioned in this Chapter, please consult Chapter 2: Materials and Methods, Section 2.2).

4.2 Primary screening

The YAC filters were probed with $^{32}$P-labelled full-length cDNAs for all five human *FMOs* simultaneously. Autoradiography gave X-ray films on which clones bearing *FMO*-like sequences could be identified. Figure 4.1 shows one such film with positive clones evident from one of the filters.

In all, sixteen positive clones were identified. However, due to the identity of one clone being ambiguous, both of its possible coordinates were noted. In total then, a request was made for seventeen YAC clones.

4.3 Preliminary characterization of requested YAC clones

Only sixteen of the seventeen requested clones were sent by the HGMP Resource Centre, due to one clone being currently unavailable (this clone being one of the two clones requested for an *FMO*-binding clone of uncertain coordinates). The names of these clones are listed in Table 4.1.

The clones were received as stabs grown on AHC agar medium and hence presented as red colonies. Glycerol stocks were immediately made for each YAC clone in duplicate.

Each of the sixteen clones were grown and subjected to a genomic DNA preparation protocol. DNA concentrations were estimated using a spectrophotometer ($A_{260}$).

DNA from these preparations was digested with the restriction enzyme EcoR1 and an agarose ‘check gel’ performed to ensure complete digestion (Figure 4.2).
Table 4.1. Names of sixteen YAC clones suspected of bearing *FMO*-like sequences. Clones were received from the UK HGMP Resource Centre following screening of the ICRF YAC human genomic DNA library with full-length human cDNA probes for all five *FMO* genes simultaneously.
Figure 4.1. Preliminary screening of ICRF YAC human genomic library for \textit{FMO} sequences. An X-ray film autoradiograph of one of fourteen nylon filters making up the ICRF YAC human genomic DNA library. Sixteen plates (96 clones/plate) are gridded in an 8x12 formation. All filters were screened simultaneously with $^{32}\text{P}$-labelled full-length cDNA probes for all five human \textit{FMO} genes (\textit{FMO1}-\textit{FMO5}) using high-stringency hybridization conditions. Here, four clones on the filter appear to contain \textit{FMO}-like sequences and were thus selected after establishing their coordinates. Non-specific binding of probe to most other clones is visible and this assists in establishing coordinates of putative positive clones.
Figure 4.2. EcoR1 digestion of yeast genomic DNA preparations bearing YACs. Shown here is a photograph of an ethidium bromide-stained 0.8% agarose gel following electrophoresis of yeast genomic DNA digested with EcoR1. Each digest contains one of sixteen YAC clones suspected of bearing human FMO genomic sequences. Order as follows: lane C, 4X58-G7; lane D, 4X64-A4; lane E, 4X74-G7; lane F, 4X78-F11; lane G, 4X79-F7; lane H, 4X86-B8; lane I, 4X86-B9; lane J, 4X86-B10; lane K, 4X86-B11; lane L, 4X86-B12; lane M, 4X134-C4; lane N, 4X135-B12; lane O, 4X136-B12; lane P, 4X148-B9; lane Q, 4Y3-C5 and lane R, HD12-H6. Lanes A and T are molecular weight standards (Gibco-BRL 1-kb ladder). Lanes B and S are empty.
Digests were then run on a suitable agarose gel and subjected to the Southern blotting protocol. The resultant nylon membrane was probed with $^{32}$P-labelled full-length cDNAs for all five human *FMO* genes simultaneously under high-stringency conditions. Autoradiography yielded an X-ray film showing fifteen of the sixteen YAC clones to contain sequences with strong sequence homology to one or more of the *FMO* genes (Figure 4.3). The one YAC clone apparently devoid of *FMO*-like sequences was one of the two YAC clones requested for one ‘positive’ of ambiguous coordinates on the primary screen. Hence, it is likely that if the other clone had been sent, rather than being unavailable, it would have revealed the presence of *FMO*-like sequences.

4.4 Assigning respective *FMO* genes to individual YAC clones

Further Southern blots were performed on the sixteen YAC clones. Once again, genomic DNA preparations were digested with EcoR1 and separated on an agarose gel before transfer. Nylon membranes generated in this manner were then probed with each of the human *FMO* cDNAs individually under high stringency conditions to see which *FMO* gene(s) were present on each of the YAC clones.

4.4i *FMO1*

Three of the YAC clones, 4X134-C4, 4X135-B12 and HD12-H6, hybridized strongly with high specificity to $^{32}$P-labelled full-length human *FMO1* cDNA probe (Figure 4.4). In each case, five DNA fragments can be seen to have hybridized with the probe and these fragments appear to be identical in size between the clones. Evidence of the high degree of probe-YAC DNA homology in these cases, in addition to the high-stringency conditions of hybridization used, exists in the fact that removal of bound probe necessitated exposure to boiling 0.5% SDS solution. Table 4.2 lists the estimated sizes of the five DNA fragments using molecular weight standards run on the same gel. Bearing in mind the large insert size of the YACs (on average, 600kb), the generation of identical ‘fingerprints’ from these clones, when digested with EcoR1 and hybridized to full-length *FMO1* cDNA, strongly suggests that the entire region of transcription for the *FMO1* gene exists within these clones. By adding up the sizes of the five fragments we can estimate this region to be around 24kb in size. In addition, it would be surprising if flanking non-coding sequences involved in regulating transcription of this gene were not also present within the clone insert (not necessarily within one or more of the hybridizing fragments, however) and therefore available for study.
Figure 4.3. Screening selected YAC clones for FMO sequences. Depicted here is an X-ray film autoradiograph generated from Southern blot analysis of yeast genomic DNA from sixteen colonies bearing YAC clones probed with full-length $^{32}$P-labelled cDNA sequences for all five FMO genes simultaneously using high stringency conditions. Clones had been selected from the ICRF YAC human genomic DNA library on suspicion of bearing FMO-like sequences. DNA was digested with EcoRI and separated through a 0.6% agarose gel before transfer. Fifteen of the clones appear to contain sequences with homology to the probe material. (A) Top half of gel. Lane 4, 4X58-G7; lane 5, 4X64-A4; lane 6, 4X74-G7; lane 7, 4X78-F11; lane 8, 4X79-F7; lane 9, 4X86-B8; lane 10, 4X86-B9; lane 11, 4X86-B10. Lanes 1 and 14, molecular weight standards (Gibco-BRL 1kb ladder) labelled with $^{32}$PdATP. Lanes 2 and 13, molecular weight standards (Gibco-BRL 1kb ladder). Lanes 3 and 12 are empty. (Gap between lanes 3 and 4 is where five lanes were excised from the nylon membrane prior to autoradiography. These lanes contained the cDNAs of each of the FMO genes as a positive control. Hybridization produced such a strong signal that it was decided to remove them.) (B) Bottom half of gel. Lane 4, 4X86-B11; lane 5, 4X86-B12; lane 6, 4X134-C4; lane 7, 4X135-B12; lane 8, 4X136-B12; lane 9, 4X148-B9; lane 10, 4Y3-C5; lane 11, HD12-H6. Lane 3 was pUC19 control. Lanes 1 and 15, molecular weight standards (Gibco-BRL 1kb ladder) labelled with $^{32}$PdATP. Lanes 2 and 14, molecular weight standards (Gibco-BRL 1kb ladder). Lanes 12 and 13 are empty. Note: Cross-hybridization of FMO probes to the molecular weight standards occurs consistently, particularly the 1.6kb fragment, perhaps because of low level contamination of cDNA samples with vector sequences or coincidental homology of sequences.
Figure 4.4. Screening selected YAC clones for the presence of \textit{FMO1} sequences. Depicted here is an X-ray film autoradiograph showing Southern blot analysis of yeast genomic DNA from sixteen YAC clones, fifteen of which are suspected of bearing human \textit{FMO}-like sequences, with $^{32}$P-labelled full-length human \textit{FMO1} cDNA as the probe. Hybridization took place under high stringency conditions. Five seemingly identical fragments appear to have bound the probe in the case of three of the clones. Order as follows: lane 3, 4X58-G7; lane 4, 4X64-A4; lane 5, 4X74-G7; lane 6, 4X78-F11; lane 7, 4X79-F7; lane 8, 4X86-B8; lane 9, 4X86-B9; lane 10, 4X86-B10; lane 11, 4X86-B11; lane 12, 4X86-B12; lane 13, 4X134-C4; lane 14, 4X135-B12; lane 15, 4X136-B12; lane 16, 4X148-B9; lane 17, 4Y3-C5; lane 18, HD12-H6. Lanes 1 and 20, molecular weight standards (Gibco-BRL 1kb ladder) labelled with $^{35}$SdATP. Lanes 2 and 19, molecular weight standards (Gibco-BRL 1kb ladder).
Table 4.2. Size and number of DNA fragments that bind human *FMO1* cDNA probe following digestion of YAC clones with EcoR1. The three YAC clones listed above are the only ones that were shown to bind *FMO1* full-length cDNA probe using high stringency conditions: thirteen other clones, twelve of which had previously been shown to bind a mixture of all five *FMO* cDNA probes when added simultaneously, failed to do so. The probe appears to bind to the same five fragments in the case of each clone. When added together, these fragments total nearly 24kb. This may be a good estimate of the size of the region transcribed for the *FMO1* gene. *Note:* size estimates were made by plotting $\log_{10}$ size of molecular weight standards (Gibco-BRL 1kb ladder) against distance travelled through gel for a standard curve, off which the distances travelled by the YAC fragments on the same gel could be read off to give $\log_{10}$ size.

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As a comparison, it has recently been estimated that the size of the rabbit \textit{FMO1} gene is about 40kb (108). However, this estimate included about 6kb of flanking DNA; in the case of our estimate of the size of the human \textit{FMO1} gene, it is not possible to say how much flanking sequence is included without further study.

4.4ii \textit{FMO2}

Ten of the YAC clones were shown to bind with high specificity to $^{32}$P-labelled full-length human \textit{FMO2} cDNA (removal of probe required boiling in 0.5% SDS solution) (Figure 4.5). The identity of these clones are as follows: 4X58-G7, 4X86-B8, 4X86-B9, 4X86-B10, 4X86-B11, 4X86-B12, 4X134-C4, 4X135-B12, 4Y3-C5 and HD12-H6. Up to five different DNA fragments from any one clone can be seen to have hybridized with the probe. Other, faint bands can be spotted, although these would appear to be the result of cross-hybridization to other sequences, in most cases probably other \textit{FMO} sequences. It seems reasonable to assume from this, and the large insert size of the YACs, that clones producing all five fragments (4X58-G7, 4X86-B8, 4X86-B9, 4X86-B10, 4X86-B11, 4X135-B12 and HD12-H6) possess the entire region of transcription for the \textit{FMO2} gene, with, in all probability, flanking promoter sequences responsible for regulating expression of the gene also present. Table 4.3 lists the estimated sizes of the DNA fragments that bind the \textit{FMO2} probe using molecular weight standards run on the same gel. From these estimates we can conclude that the genomic region of \textit{FMO2} transcription is about 27kb in size. Three of the clones (4X58-G7, 4X134-C4 and 4Y3-C5) failed to produce all five fragments and so we can assume that they contain only partial regions of the \textit{FMO2} gene. This is discussed further later.

4.4iii \textit{FMO3}

Nine of the YAC clones were shown to bind with high specificity to $^{32}$P-labelled full-length human \textit{FMO3} cDNA (removal of probe required boiling in 0.5% SDS solution) (Figure 4.6). The identity of these clones are as follows: 4X79-F7, 4X86-B8, 4X86-B9, 4X86-B10, 4X86-B11, 4X86-B12, 4X135-B12, 4Y3-C5 and HD12-H6. Six, perhaps seven, different DNA fragments from any one clone can be seen to have hybridized with the probe. Some other faint bands can be seen but these, after careful analysis, were dismissed and assumed to be the result of cross-hybridization to related sequences or partially digested fragments. Apart from these 'spurious' bands and the six (or seven) 'genuine' \textit{FMO3}-binding fragments, bands of any other size are not visible.
Figure 4.5. Screening YAC clones for the presence of FMO2 sequences. Depicted here is an X-ray film autoradiograph showing Southern blot analysis of yeast genomic DNA from sixteen YAC clones, fifteen of which are suspected of bearing human FMO-like sequences, with $^{32}$P-labelled full-length human FMO2 cDNA as the probe. Hybridization took place under high stringency conditions. Up to five different fragments appear to contain sequences that bind the probe in the case of ten of the clones. Other, faint visible fragments appear the result of cross-hybridization to other sequences, some of which are probably other FMO sequences. Order as follows: lane 3, 4X58-G7; lane 4, 4X64-A4; lane 5, 4X74-G7; lane 6, 4X78-F11; lane 7, 4X79-F7; lane 8, 4X86-B8; lane 9, 4X86-B9; lane 10, 4X86-B10; lane 11, 4X86-B11; lane 12, 4X86-B12; lane 13, 4X134-C4; lane 14, 4X135-B12; lane 15, 4X136-B12; lane 16, 4X148-B9; lane 17, 4Y3-C5; lane 18, HD12-H6. Lanes 1 and 20, $^{32}$PdATP-labelled molecular weight standards (Gibco-BRL 1kb ladder).
**Table 4.3.** Size and number of DNA fragments that bind human *FMO2* cDNA probe following digestion of YAC clones with EcoRl. The ten YAC clones listed above are those that were shown to bind *FMO2* full-length cDNA probe using high stringency hybridization conditions. Six other YAC clones, five of which had previously been shown to bind a mixture of all five *FMO* cDNA probes when added simultaneously, failed to do so. The probe appears to bind up to five different fragments in the case of any one clone. Across all clones, only these five fragments appear to have hybridized to the probe. When added together, these five fragments total about 27kb: we can presume this an estimate of the size of the genomic region transcribed for the *FMO2* gene. Hence, seven of the clones listed possess this region entirely whereas three possess only a portion. *Note:* size estimates were made by plotting log_{10} size of molecular weight standards (Gibco-BRL 1kb ladder) against distance travelled through gel for a standard curve, off which the distances travelled by the YAC fragments on the same gel could be read off to give log_{10} size.

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Figure 4.6. Screening YAC clones for the presence of FMO3 sequences. Depicted here is an X-ray film autoradiograph showing Southern blot analysis of yeast genomic DNA from sixteen YAC clones, fifteen of which are suspected of bearing human FMO-like sequences, with \(^{32}\text{P}\)-labelled full-length human FMO3 cDNA as the probe. Hybridization took place under high stringency conditions. Six, possibly seven, different fragments appear to bind the probe in the case of ten of the clones, although other seemingly spurious faint bands can be seen in places. Order as follows: lane 3, 4X58-G7; lane 4, 4X64-A4; lane 5, 4X74-G7; lane 6, 4X78-F11; lane 7, 4X79-F7; lane 8, 4X86-B8; lane 9, 4X86-B9; lane 10, 4X86-B10; lane 11, 4X86-B11; lane 12, 4X86-B12; lane 13, 4X134-C4; lane 14, 4X135-B12; lane 15, 4X136-B12; lane 16, 4X148-B9; lane 17, 4Y3-C5; lane 18, HD12-H6. Lanes 2 and 19, molecular weight standards (Gibco-BRL 1kb ladder). Lanes 1 and 20, \(^{35}\text{S}\)S\(\text{dATP}\)-labelled molecular weight standards (Gibco-BRL 1kb ladder).
It seems reasonable to assume from this, and the large insert size of the YACs, that the nine clones possess the entire region of transcription for the *FMO3* gene, with, in all probability, flanking promoter sequences responsible for regulating expression of the gene also present. Table 4.4 lists the estimated sizes of the DNA fragments that bind the *FMO3* probe using molecular weight standards run on the same gel and allows us to estimate the genomic region of *FMO3* transcription to be between about 38 and 55kb in size. Work by Dolphin and coworkers (134) has indicated the minimum size of the human *FMO3* gene to be 22.5kb.

4.4iv *FMO4*

Three of the YAC clones, 4X134-C4, 4X135-B12 and HD12-H6, hybridized strongly with high specificity to $^{32}$P-labelled full-length human *FMO4* cDNA probe (Figure 4.7). In each case, four DNA fragments are identifiable as binding this probe and being specific for the *FMO4* gene: repeated washes with boiling 0.5% SDS solution were required to completely remove probe from these four fragments. Other, faint bands visible after longer exposures appear to be the result of cross-hybridization with *FMO1* sequences (it so happens that the same three clones bind both *FMO1* and *FMO4* probe, see Table 4.2). It can be seen that the four *FMO4*-binding fragments migrate for identical distances in the case of each of the three clones and that by using molecular weight standards run on the same gel an estimate of their sizes can be given (Table 4.5). Given the large insert size of the YACs (on average, 600kb), the generation of identical ‘fingerprints’ from these clones, when digested with EcoR1 and hybridized to full-length *FMO4* cDNA, suggests strongly that the entire genomic region of transcription for the *FMO4* gene exists within these clones. By adding up the size of all four fragments we can estimate this region to be around 36kb in size. In addition, it would be surprising if flanking non-coding sequences involved in regulating transcription of this gene were not also present and therefore available for study.

4.4v *FMO5*

Four of the YAC clones, 4X64-A4, 4X74-G7, 4X78-F11 and 4X148-B9, hybridized strongly with high specificity to $^{32}$P-labelled full-length human *FMO5* cDNA probe when using high-stringency hybridization conditions (Figure 4.8). In each case, five DNA fragments can be seen to have hybridized with the probe and these fragments appear to be identical in size between the clones. Repeated washes with boiling 0.5% SDS solution were required to remove probe from these fragments.
Table 4.4. Size and number of DNA fragments that bind human *FMO3* cDNA probe following digestion of YAC clones with EcoR1. The ten YAC clones listed above are the those that were shown to bind *FMO3* full-length cDNA probe using high stringency hybridization conditions. Six other YAC clones, five of which had previously been shown to bind a mixture of all five *FMO* cDNA probes when added simultaneously, did not hybridize to the probe. The probe appears to bind seven different fragments in the case of any one clone, although there is some doubt over whether fragment 2 is a genuine, separate *FMO3*-binding fragment (hence it has been marked with an asterisk). Across all clones, these seven fragments appear to be orthologous and, when added together, total about 54.5kb (37.8kb if we ignore fragment 2): we can presume this an estimate of the size of the genomic region transcribed for the *FMO3* gene. Note: size estimates were made by plotting log_{10} size of molecular weight standards (Gibco-BRL 1kb ladder) against distance travelled through gel for a standard curve, off which the distances travelled by the YAC fragments on the same gel could be read off to give log_{10} size.
Figure 4.7. Screening selected YAC clones for the presence of *FMO4* sequences. Depicted here is an X-ray film autoradiograph showing Southern blot analysis of yeast genomic DNA from sixteen YAC clones, fifteen of which are suspected of bearing human *FMO*-like sequences, with $^{32}$P-labelled full-length human *FMO4* cDNA as the probe. Hybridization took place under high stringency conditions. Four separate fragments appear to have bound the probe in the case of three of the clones and their positions have been marked by arrows owing to the molecular weight standards not being visible in this case. The other faint bands that can be seen were ruled out because they were shown to have hybridized far more strongly to other *FMO* probes, and so are likely the result of cross-hybridization between *FMO* sequences, probably in those regions showing greatest conservation between all five forms. Order as follows: lane 3, 4X58-G7; lane 4, 4X64-A4; lane 5, 4X74-G7; lane 6, 4X78-F11; lane 7, 4X79-F7; lane 8, 4X86-B8; lane 9, 4X86-B9; lane 10, 4X86-B10; lane 11, 4X86-B11; lane 12, 4X86-B12; lane 13, 4X134-C4; lane 14, 4X135-B12; lane 15, 4X136-B12; lane 16, 4X148-B9; lane 17, 4Y3-C5; lane 18, HD12-H6. Lanes 2 and 19, molecular weight standards (Gibco BRL 1kb ladder). Lanes 1 and 20, molecular weight standards (Gibco-BRL 1kb ladder) labelled with $^{35}$SxdtATP (not easily visible on this particular film due to a very short exposure time of less than 24 hours).
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<td></td>
<td>Total</td>
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Table 4.5. Size and number of DNA fragments that bind human FMO4 cDNA probe following digestion of YAC clones with EcoR1. Above are listed three YAC clones that were shown to bind FMO4 full-length cDNA probe using high stringency hybridization conditions. Thirteen other clones, twelve of which had previously been shown to bind a mixture of all five FMO cDNA probes when added simultaneously, failed to hybridize to the probe. The probe appears to bind four different fragments in the case of any one of the three clones listed. Across all clones, these four fragments appear to be orthologous and, when added together, total about 36kb. This provides an estimate of the size of the genomic region transcribed for the FMO4 gene. Note: size estimates were made by plotting log_{10} size of molecular weight standards (Gibco-BRL 1kb ladder) against distance travelled through gel for a standard curve, off which the distances travelled by the YAC fragments on the same gel could be read off to give log_{10} size.
Figure 4.8. Screening selected YAC clones for the presence of $FM05$ sequences. Depicted here is an X-ray film autoradiograph showing Southern blot analysis of yeast genomic DNA from sixteen YAC clones, fifteen of which are suspected of bearing human $FMO$-like sequences, with $^{32}$P-labelled full-length human $FM05$ cDNA as the probe. Hybridization took place under high stringency conditions. Seven different fragments bind the probe in the case of four of the clones. Order as follows: lane 3, 4X58-G7; lane 4, 4X64-A4; lane 5, 4X74-G7; lane 6, 4X78-F11; lane 7, 4X79-F7; lane 8, 4X86-B8; lane 9, 4X86-B9; lane 10, 4X86-B10; lane 11, 4X86-B11; lane 12, 4X86-B12; lane 13, 4X134-C4; lane 14, 4X135-B12; lane 15, 4X136-B12; lane 16, 4X148-B9; lane 17, 4Y3-C5; lane 18, HD12-H6. Lanes 1 and 20, molecular weight standards (Gibco-BRL 1kb ladder) labelled with $^{35}$S$ackslash$dATP. Lanes 2 and 19 are empty.
Table 4.6. Size and number of DNA fragments that bind human *FM05* cDNA probe following digestion of YAC clones with EcoR1. The four YAC clones that were shown to bind *FM05* full-length cDNA probe under high-stringency hybridization conditions are listed above. Twelve other clones, eleven of which had previously been shown to bind a mixture of all five *FMO* cDNA probes when added simultaneously failed to do so. The probe appears to bind to the same seven fragments in the case of each clone. When added together these fragments total 34kb, which may be a good estimate of the size of the region of transcription of the *FM05* gene. Note: size estimates were made by plotting log$_{10}$ size of molecular weight standards (Gibco-BRL 1kb ladder) against distance travelled through gel for a standard curve, off which the distances travelled by the YAC fragments on the same gel could be read off to give log$_{10}$ size.
Allowing for the large insert size of the YACs (on average, 600kb), the generation of identical ‘fingerprints’ from these clones, when digested with EcoR1 and hybridized to full-length FMO5 cDNA, strongly suggests that the entire region of transcription for the FMO5 gene exists within these clones. Once more, it would be surprising if flanking non-coding sequences involved in regulating transcription of this gene were not also present and therefore available for study. Table 4.6 lists the estimated sizes of the five DNA fragments using molecular weight standards run on the same gel. Together, these fragments total 34kb in size: we can take this to be an estimate of the size of the genomic region of transcription for the FMO5 gene.

4.5 Summary

Southern blotting analysis of the sixteen YAC clones isolated from the ICRF human genomic library reveals that fifteen have sequences that hybridize very strongly to human FMO cDNA sequences.

Screening each of these clones with individual members of the FMO gene family appears to reveal that three contain FMO1 sequences, ten FMO2 sequences, ten FMO3 sequences, three FMO4 sequences and four FMO5 sequences. Table 4.7 lists the FMO genes that appear to reside on each clone. Interestingly, two clones (4X135-B12 and HD12-H6) appear to have FMO1, FMO2, FMO3 and FMO4 genes, furthering the hypothesis of a gene cluster. However, the FMO5 gene only appears in isolation (4X64-A4, 4X74-G7, 4X78-F11 and 4X148-B9), perhaps suggesting that this member is removed from the other four members of the family. Further cursory analysis reveals that the two clones possessing FMO1, FMO2, FMO3 and FMO4 genes, originate from two separate donors within the library (three lymphoblastoid cell lines were used to construct the library, two female [4X and HD clones] and one male [4Y clones]).

Furthermore, it becomes apparent from logical deduction that the data presented here indicates the gene order along chromosome 1q for FMO1, FMO2, FMO3 and FMO4 to be either 1423 or 4123 (in either orientation). Of all five of the FMO genes, only FMO2 appears to be incomplete on any of the clones, these being 4X58-G7, 4X134-C4 and 4Y3-C5 (Table 4.7). If we examine Table 4.3 and Figure 4.6 we gain an insight into the proportions of the FMO2 gene present on these clones in terms of the number of EcoR1 FMO-binding fragments that were identified in each case. Closer examination of this data confirms the postulated 1423 or 4123 gene order when one considers the other
*FMO* genes that were shown to be present on these three clones. It is not obvious, however, why only *FMO2* appears in a truncated form on any of the clones. Most likely, this was simply a chance event.

The isolation of four of the five known members of the human *FMO* gene family on single YAC clones provides an excellent opportunity for characterizing these genes and how they are regulated.
<table>
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Table 4.7. Human *FMO* gene family members present within sixteen YAC clones. Clones were from the ICRF human genomic DNA library and were characterized by the Southern blotting technique. +; this gene was found to be present, probably in its entirety with respect to the region of transcription. +p; this gene was found to be present but not in its entirety with respect to the region of transcription. -; this gene was found not to be present.
Chapter 5

Fluorescence *in-situ* hybridization analysis of YAC clones bearing human genomic sequences containing *FMO* genes.
5.1 Background

Chapter 4 (Isolation of YACs bearing FMO-containing human genomic inserts) details the outcome of screening an ICRF yeast artificial chromosome (YAC) human genomic DNA library for the presence of FMO gene sequences. Sixteen clones were selected. Of these, fifteen were shown by Southern hybridization analysis to contain within their inserts the sequences of one or more of the FMO genes (Chapter 4, Table 4.7). Having isolated these clones and established which FMO genes were present within their inserts, it was decided they could be used to further knowledge regarding the chromosomal localization of human FMO gene family members. Already I have shown (Chapter 3: Localization of human FMO2 and FMO5 genes to chromosome 1q) that the human FMO2 and FMO5 genes reside on the long arm of chromosome 1. This complemented previous research showing that the human FMO1 (104), FMO3 (171) and FMO4 (84) genes also reside on chromosome 1q. Furthermore, FMO1 has been further localized to 1q23-25 (171) by in-situ hybridization of human metaphase chromosomes. Of further relevance, it was shown in Chapter 4 (see Table 4.7) that two of the YAC clones have FMO1, FMO2, FMO3 and FMO4 genes within their inserts. Clones bearing FMO5, however, did not contain any of the other FMO genes. Hence, it may be that FMO1, FMO2, FMO3 and FMO4 genes are clustered, with FMO5 removed to another location on 1q.

I set out to demonstrate which region of the human genome the inserts of the YAC clones originated from using the fluorescence in-situ hybridization (FISH) technique. This technique would also potentially demonstrate the genomic location of the respective FMO genes contained within these inserts to a more exact degree than simply 1q in the case of FMO2, FMO3, FMO4, FMO5 and possibly even refine the 1q23-25 location of FMO1. Overall, the data may assist in establishing whether some or all members of the FMO gene family exist in a clustered formation, and whether FMO5 resides at a location distant from the other four genes, as the Southern hybridization analysis of the clones would suggest.
Figure 5.1. Human metaphase chromosome spread following Giemsa (G)-banding.
Specific details regarding the methodology employed in FISH analysis are given in Chapter 2 (Materials and Methods), Section 2.3. Briefly, the procedure involves the preparation of human metaphase chromosome spreads (Figure 5.1) to which are hybridized biotin-labelled probes, in this case YACs. The position of the hybridization signal indicates where the probe has bound to a complimentary sequence on the chromosome(s), the location of which can be measured in terms of the corresponding chromosome Giemsa (G) band.

Furthermore, FISH provides an excellent method of establishing whether a given YAC insert is chimeric. Chimeric YAC clones, which represent the most common artifact in human YAC libraries, are those containing two or more segments of noncontiguous DNA. The ICRF YAC library used here has an estimated chimerism frequency of up to 30%. The formation of chimeric YACs is likely due to coligation events but may also result from recombination of YACs after introduction into yeast spheroplasts. Alternatively, cotransformation events may result in the introduction and stable independent maintenance of two or more YACs in the same cell. If a YAC clone is chimeric then it will hybridize to two or more independent locations in the human genome.

The outcome of FISH analysis on each of the sixteen YAC clones will be considered in turn.

5.2 4X58-G7

This clone hybridized strongly to 1q23-24, 1p36, 5q33-34, Xq13 and the centromeric region of chromosome 11 (Figure 5.2), and is therefore probably chimeric (see 'Note' at end of Section 5.18, however). Table 4.7 in Chapter 4 shows that Southern hybridization experiments identified the presence of part of the $FMO2$ gene within this clone. Bearing in mind that $FMO2$ was found to be present on three other clones also containing $FMO1$, (4X134-C4, 4X135-B12 and HD12-H6) which, as we have already stated, has been localized to 1q23-25 by in situ-hybridization, it seems likely that $FMO2$ sequences within this clone were responsible for the hybridization signal at 1q23-24. The results for the other $FMO2$-containing clones (4X86-B8, 4X86-B9, 4X86-B10, 4X86-B11, 4X86-B12, 4X134-C4, 4X135-B12, 4Y3-C5 and HD12-H6) would seem to confirm this.
Figure 5.2. FISH analysis of YAC clone 4X58-G7. Regions to which YAC clone 4X58-G7 hybridizes on human metaphase chromosomes can be seen (green areas). The clone is chimeric and hybridizes strongly to 1q23-24 (arrowed), 1p36, 5q33-34 and Xq13, as well as the centromeric region of chromosome 11.
5.3 4X64-A4

A strong signal was evident at 1q21 and weaker ones at 1p36 and 20q11, (Figure 5.3). YAC clone 4X64-A4 was shown by Southern hybridization analysis to contain *FM05* only (Chapter 4). As we know from PCR analysis of human-rodent somatic cell hybrids that *FM05* resides on 1q (Chapter 3, Sections 3.3 and 3.4ii), the signal at 1q21 is likely the result of the presence of this gene within the insert. The FISH data of other clones bearing *FM05* (4X74-G7, 4X78-F11 and 4X148-B9) would seem to confirm this.

5.4 4X74-G7

The only definite single area of hybridization for this clone was at 1q21 (Figure 5.4). 4X74-G7 was previously shown to possess only *FM05* (Chapter 4).

5.5 4X78-F11

A clear hybridization signal was detected at 1q21 (Figure 5.5). 4X78-F11 was another clone shown to contain *FM05* only (Chapter 4).

5.6 4X79-F7

Although possibly chimeric, this clone hybridized strongly to 1q23-24 and only very weakly to 8q21 (Figure 5.6). 4X79-F7 was shown to possess only *FM03* (Chapter 4). Note that 1q23-24 is the region that was postulated to have hybridized to the *FM02* region of 4X58-G7.

5.7 4X86-B8

A strong hybridization signal was located at 1q23-24 only (Figure 5.7). 4X86-B8 was shown to possess *FM02* and *FM03* genes (Chapter 4).
Figure 5.3. FISH analysis of YAC clone 4X64-A4. Regions to which YAC clone 4X64-A4 hybridizes on human metaphase chromosomes can be seen (green areas). The clone is chimeric, hybridizing strongly to 1q21 (arrowed) and weakly to 1p36 and 20q11.
Figure 5.4. FISH analysis of YAC clone 4X74-G7. Regions to which YAC clone 4X74-G7 hybridizes on human metaphase chromosomes can be seen (green areas). The clone hybridizes strongly to 1q21 (arrowed).
Figure 5.5. FISH analysis of YAC clone 4X78-F11. Regions to which YAC clone 4X78-F11 hybridizes on human metaphase chromosomes can be seen (green areas). The clone hybridizes strongly to 1q21 (arrowed).
Figure 5.6. FISH analysis of YAC clone 4X79-F7. Regions to which YAC clone 4X79-F7 hybridizes on human metaphase chromosomes can be seen (green areas). The clone is chimeric, hybridizing strongly to 1q23-24 (arrowed) and very faintly to 8q21.
Figure 5.7. FISH analysis of YAC clone 4X86-B8. Regions to which the YAC clone 4X86-B8 hybridizes to on human metaphase chromosomes can be seen (green areas). The clone hybridizes strongly to 1q23-24 (arrowed) only.
5.8 4X86-B9

The only definite signal arises from the region 1q23-24 (Figure 5.8). 4X86-B8 was found to possess \(FMO2\) and \(FMO3\) (Chapter 4).

5.9 4X86-B10

The region of hybridization is concentrated at 1q23-24 (Figure 5.9). 4X86-B10 was shown to contain \(FMO2\) and \(FMO3\) (Chapter 4).

5.10 4X86-B11

A strong signal is evident at 1q23-24 (Figure 5.10). 4X86-B11 was found to contain \(FMO2\) and \(FMO3\) (Chapter 4).

5.11 4X86-B12

Hybridization is confined to 1q23-24 (Figure 5.11). 4X86-B12 was shown to possess \(FMO2\) and \(FMO3\) (Chapter 4).

5.12 4X134-C4

A strong hybridization signal is located at 1q23-24 (Figure 5.12). 4X134-C4 was found to contain \(FMO1, FMO2\) (partial) and \(FMO4\) (Chapter 4). The presence of the \(FMO1\) gene within the insert of this clone is significant as it would indicate that the chromosomal location of this gene can be refined from 1q23-25 to the more precise region 1q23-24.

5.13 4X135-B12

A strong hybridization signal is located at 1q23-24 (Figure 5.13). 4X135-B12 was shown to possess \(FMO1, FMO2, FMO3\) and \(FMO4\) (Chapter 4).
Figure 5.8. FISH analysis of YAC clone 4X86-B9. Regions to which YAC clone 4X86-B9 hybridizes to on human metaphase chromosomes can be seen (green areas). The clone hybridizes strongly to 1q23-24 (arrowed) only.
Figure 5.9. FISH analysis of YAC clone 4X86-B10. Regions to which the YAC clone 4X86-B10 hybridizes to on human metaphase chromosomes can be seen (green areas). The clone hybridizes strongly to 1q23-24 (arrowed) only.
Figure 5.10. FISH analysis of YAC clone 4X86-B11. Regions to which the YAC clone 4X86-B11 hybridizes to on human metaphase chromosomes can be seen (green areas). The clone hybridizes strongly to 1q23-24 (arrowed) only.
Figure 5.11. FISH analysis of YAC clone 4X86-B12. Regions to which the YAC clone 4X86-B12 hybridizes to on human metaphase chromosomes can be seen (green areas). The clone hybridizes strongly to 1q23-24 (arrowed) only.
Figure 5.12. FISH analysis of YAC clone 4X134-C4. Regions to which the YAC clone 4X134-C4 hybridizes to on human metaphase chromosomes can be seen (green areas). The clone hybridizes strongly to 1q23-24 (arowed) only.
Figure 5.13. FISH analysis of YAC clone 4X135-B12. Regions to which the YAC clone 4X135-B12 hybridizes to on human metaphase chromosomes can be seen (green areas). The clone hybridizes strongly to 1q23-24 (arrowed) only.
5.14 4X136-B12

A signal was apparent at 9p22-23 (Figure 5.14). 4X136-B12 was found to contain none of the *FMO* genes (Chapter 4).

5.15 4X148-B9

This is possibly a chimeric clone. Strong signals are apparent at 1q21 and 8p21, with weaker signals at 1p36, 1p12-13 and 16q22 (Figure 5.15). 4X148-B9 was shown to possess only *FMO5* (Chapter 4).

5.16 4Y3-C5

This clone hybridizes to 1q23-24 (Figure 5.16). 4Y3-C5 contains *FMO2* (partial) and *FMO3* (Chapter 4).

5.17 HD12-H6

A possibly chimeric clone, hybridizing strongly to 1q23-24 and more faintly to 7q34 (Figure 5.17). HD12-H6 was shown to have *FMO1, FMO2, FMO3* and *FMO4* genes (Chapter 4).

5.18 Implications

Table 5.1 summarizes the above data and indicates the chromosomal location of each YAC clone alongside the *FMO* genes it contains.

It would seem that the *FMO1, FMO2, FMO3* and *FMO4* genes are clustered as they all exist together on two of the clones and any clone bearing one or more of these genes is found to hybridize at 1q23-24 (some clones are quite possibly chimeric, but 1q23-24 is the only common denominator for all of them). Thus, I have further refined the chromosomal localization of these genes to 1q23-24 (Table 5.2 and Figure 5.18).
Figure 5.14. FISH analysis of YAC clone 4X136-B12. Regions to which the YAC clone 4X136-B12 hybridizes to on human metaphase chromosomes can be seen (green areas). The clone hybridizes strongly to 9p22-23 (arrowed) only.
Figure 5.15. FISH analysis of YAC clone 4X148-B9. Regions to which YAC clone 4X148-B9 hybridizes to on human metaphase chromosomes can be seen (green areas). This clone is highly chimeric, hybridizing strongly to 1q21 (arrowed) and 8p21, and more faintly to 1p36, 1p12-13 and 16q22.
Figure 5.16. FISH analysis of YAC clone 4Y3-C5. Regions to which the YAC clone 4Y3-C5 hybridizes to on human metaphase chromosomes can be seen (green areas). The clone hybridizes strongly to 1q23-24 (arrowed) only.
Figure 5.17. FISH analysis of YAC clone HD12-H6. Regions to which the YAC clone HD12-H6 hybridizes to on human metaphase chromosomes can be seen (green areas). The clone hybridizes strongly to 1q23-24 (arrowed) and faintly to 7q34.
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<th>YAC Clone</th>
<th>FMO Gene Present</th>
<th>Chromosomal Location</th>
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<tr>
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<td>HD12-H6</td>
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<td>1q23-24 7q34</td>
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</table>

Table 5.1. Human FMO gene content of sixteen YAC clones and their chromosomal localization. The FMO gene content of clones was determined by Southern blotting and the chromosomal localization of their inserts determined by fluorescence in-situ hybridization to human metaphase chromosomes. +; gene present. +p; only part of gene present. -; gene not present. When chimeric, the chromosomal location thought to be derived from the particular FMO gene(s) present is given first in larger type. Locations in bold (including all 1q23-24 and 1q21 regions) signify strong hybridization signals. c, centromeric region.
*FMO5*, on the other hand, appears to be located several megabases away from these four genes towards the centromere of chromosome 1. We can say this because the four YAC clones bearing *FMO5* hybridize to 1q21 (two are possibly chimeric but 1q21 is the only common denominator) and contain none of the other *FMO* genes (Table 5.2 and Figure 5.18).

We can only speculate at this stage as to the significance of these findings and the implications they may have regarding how this gene family evolved and the regulation of expression of its members. For example, it is intriguing that *FMO5* appears to be separated by some distance from the other four members of the family. It is tempting to speculate whether other hitherto undiscovered *FMO* genes exist in the region between 1q23-24 and 1q21.

**Note:** It is possible that the degree of chimerism of some of the YAC clones has been exaggerated. We know from FISH analysis of cosmid clones containing human genomic inserts with sequence homology to *FMO* sequences that regions of the genome exist capable of hybridizing strongly to *FMO* sequences (Chapter 8: Analysis of cosmid clones isolated from a chromosome 1-specific library by fluorescence in-situ hybridization to human metaphase chromosomes), in particular, centromeric and telomeric regions. Thus, it may have been that some of the YACs containing *FMO* genes were cross-hybridizing to these other regions of the genome, rather than actually containing them as chimeras. For example, if one compares Table 5.1 in this Chapter with Table 8.1 in Chapter 8, three YAC clones and three cosmid clones hybridize to 1p36: it would seem extremely unlikely that this could be a mere coincidence.
Table 5.2. Chromosomal localization of the five known members of the human *FMO* gene family. Established through performing fluorescence *in-situ* hybridisation to Giemsa (G)-banded human metaphase chromosomes, with YAC clones bearing one or more of the *FMO* genes as probes.
Figure 5.18. Location of FMO genes on human chromosome 1. A combination of PCR analysis of human-rodent somatic cell hybrids and fluorescence in-situ hybridisation to human metaphase chromosomes using FMO-containing YACs as probes, shows FMO5 to reside at 1q21 and FMOs1,2,3&4 to reside at 1q23-24. Shown here is a diagrammatic representation of human chromosome 1, Giemsa (G)-banded at metaphase.
Chapter 6

Pulsed field gel electrophoresis of YAC clones bearing human \textit{FMO} sequences
6.1 Background

In Chapters 4 and 5 (Isolation of YACs bearing FMO-containing human genomic inserts and Fluorescence in-situ hybridization analysis of YAC clones bearing human genomic sequences containing FMO genes, respectively), I detail how a number of YAC clones isolated from a human genomic library with inserts containing one or more of the FMO genes were subjected to fluorescence in-situ hybridization (FISH) to human metaphase chromosomes. This allowed me to establish a further resolution in the chromosome location of the five known FMO genes.

Further characterization of these YACs could start with sizing of their inserts, known to be 600kb on average. After this, work could move on to, for example, restriction mapping of the inserts in conjunction with Southern hybridization studies to deduce the unequivocal gene order of FMO1, FMO2, FMO3 and FMO4 along the chromosome. Because of the large insert sizes of the YACs, this process would most easily be undertaken, at least in the first instance, through the use of so-called 'rare-cutter' restriction enzymes, thereby generating fewer, larger DNA fragments. However, analysis of such large restriction fragments, never mind intact YACs to determine insert size, could not be properly undertaken with conventional gel electrophoresis, as resolution of fragments with this technique cannot be obtained at much more than 50kb. The development of pulsed field gel electrophoresis (PFGE), however, has allowed the resolution of fragments up to 10mb in size (345, 346) (the size of an average human metaphase chromosome band). The precise molecular mechanism underlying how PFGE surpasses the resolving capacity of conventional electrophoresis twenty-fold is not totally agreed upon and would be outside the scope of this section anyway. However, the underlying principle of the technique, of which have arisen numerous variations, is that DNA molecules within the agarose gel are subjected to electric fields that continually change direction. This is achieved by the design of electrophoresis apparatus with numerous electrodes set at different angles to the gel. DNA molecules are forced to perpetually reorientate themselves within the agarose gel each time the direction of the electric field is changed. In conventional gel electrophoresis, the direction of the electric field is constant, so this reorientation factor does not apply. Small molecules reorientate faster than larger ones (347) and this is believed to result in the dramatic increase in resolving capacity. PFGE protocols have evolved which differ according to the angle of the changing electric fields and the length of time that each field is maintained before its direction is altered.
('pulse time'), which can vary from between 0.1 to 1000s depending on the sizes of the DNA molecules being separated.

Here, I detail the use of PFGE to size the inserts of selected YAC clones previously shown to contain FMO genes.

6.2 PFGE to determine YAC insert size

Three YAC clones were chosen to undergo PFGE analysis; 4X74-G7, 4X78-F11 and 4X135-B12. Clones 4X74-G7 and 4X78-F11 contain FMO5, localize to 1q21 and appear not to be chimeric, whereas clone 4X135-B12 contains FMO1, FMO2, FMO3 and FMO4, localizes to 1q23-24 and is also apparently non-chimeric (Chapters 4 and 5).

A brief overview will be given here of the steps involved in preparing and running the PFGE. First steps entail encasing the yeast cells (as spheroplasts) in agarose moulds. Only then are the cells lysed and treatment started to remove proteins and other cellular debris. Isolating genomic DNA in this manner prior to PFGE prevents the shearing of intact chromosomes, something that is prone to occur when performing conventional DNA preparations in free solution. A more detailed account of the procedures involved can be found in Chapter 2 (Materials and Methods), Section 2.4.

Direct from glycerol stocks, YAC clones 4X74-G7, 4X78-F11 and 4X135-B12 were spread on AHC agar plates. AHC is a complete medium for growing the Saccharomyces cerevisiae host strain AB1380 while selecting for both arms of the vector pYAC4. Colonies appear on the plate coloured white if the human genomic insert has been lost, or red if it has been retained.

Individual red colonies for each of the three clones were picked and used to inoculate AHC media. After three days of growth, the cultures were centrifuged and pellets washed. Having estimated cell density with a spectrophotometer (A600), spheroplasts were formed by the addition of lyticase enzyme.

The spheroplast cell suspension was then mixed with 1% agarose (low melting point variety designed specifically for PFGE) before pouring into well moulds. After setting, the agarose blocks underwent a series of incubations to lyse the spheroplasts, digest released proteins (proteinase K incubation) and wash away remaining cellular debris. An incubation step with RNase A to remove RNA was
also included. Finally, the blocks were stored in EDTA solution, at which point it was assumed that only intact chromosomal DNA remained in the agarose.

Two days prior to PFGE, the DNA blocks were repeatedly washed with TE solution before cutting into three suitably sized pieces ready for insertion into the wells of the pulse field agarose gel. The latter was made from the same PFGE agarose used to make the DNA blocks, this time at 1.5% concentration. Figure 6.1 is a photograph of a pulse field gel on which were loaded three agarose block pieces each of 4X74-G7, 4X78-F11 and 4X135-B12. Another YAC clone isolated from a different library and not shown to contain any FMO genes within its human genomic DNA insert was loaded to serve as a negative control in the event of subsequent Southern hybridization. Also loaded on the gel were two types of marker: concatameric λ DNA molecules (48.5kb each) giving up to 21 visible fragments ranging from 48.5-1019kb, and the sixteen chromosomes of S. cerevisiae (strain YPH80) ranging in size from 225-2200kb.

Examination of the gel shown in Figure 6.1 immediately reveals the YAC of clone 4X135-B12 as an extra chromosome band (lanes d, e and f, position marked by an arrow). From this gel, we can estimate that the YAC is in the region of 480kb in size. It follows that this is also a reliable estimate of the size of the human genomic insert, given that the two arms of the YAC amount to less than 10kb.

Regarding YAC clones 4X78-F11 and 4X74-G7, we cannot see a YAC separate from the other chromosomes of the host (Figure 6.1, lanes h, i and j, and lanes l, m and n, respectively). This usually indicates that the YAC is of a size very similar to one of the native chromosomes (doublets are not always easy to spot in these circumstances).

The control YAC clone 3008 was already known to be in the order 240kb in size and can just be seen to have migrated slightly more slowly than the 225kb native chromosome (Figure 6.1, lanes p, q and r).
Figure 6.1. Pulse field gel electrophoresis of genomic DNA preparations from yeast clones bearing yeast artificial chromosomes. Shown here is a photograph of an agarose gel that has undergone pulse field gel electrophoresis (PFGE) and subsequent staining with ethidium bromide. Samples loaded on the gel include genomic DNA preparations from yeast clones bearing yeast artificial chromosomes (YACs) with human genomic inserts, some of which have previously been shown to contain FMO genes. Lane order as follows: Lanes a and t, λ Pulse Markers (Sigma) consisting of λ DNA concatemers (1-21) to give fragments ranging from 48.5-1019kb; Lanes b and s, whole intact yeast chromosomes from S. cerevisiae strain YPH80 (Sigma), sixteen chromosomes in total* ranging in size from 225-2200kb; lanes d, e and f, YAC clone 4X135-B12, previously shown to have a non-chimeric insert containing FMO1, FMO2, FMO3 and FMO4 (YAC is clearly visible as an extra chromosome and its position has been marked by an arrow); lanes h, i and j, YAC clone 4X78-F11, shown previously to bear a non-chimeric insert containing FMO5; lanes l, m and n, YAC clone 4X74-G7, also shown to have a non-chimeric insert bearing FMO5; lanes p, q and r, YAC clone 3008, bearer of a human genomic insert without FMO genes; lanes c, g, k and o are empty.

*, the chromosome numbers and their sizes are given as follows in order of decreasing electrophoretic mobility through the gel: Fragment 1 - chromosome 1, 225kb; Fragment 2 - chromosome 6, 295kb; Fragment 3 - chromosome 3, 375kb; Fragment 4 - chromosome 9, 450kb; Fragment 5 - chromosome 8, 555kb; Fragment 6 - chromosome 5, 610kb; Fragment 7 - chromosome 11, 680kb; Fragment 8 - chromosome 10, 745kb; Fragment 9 - chromosome 14, 785kb; Fragment 10 - chromosome 2, 815kb; Fragment 11 - chromosome 13, 915kb; Fragment 12 - chromosome 16, 945kb; Fragment 13 - chromosome 15, 1100kb; Fragment 13 - chromosome 7, 1120kb (chromosomes 15 and 7 migrate as a doublet band); Fragment 14 - chromosome 4, 1640kb and; Fragment 15 - chromosome 12, 2200kb.
6.3 Southern blotting of the pulsed field gel

To identify the presence of the two YACs not visible on the agarose gel, and to confirm the identity of all three YACs with regard to FMO content, Southern hybridization was undertaken. (If the inserts of the YACs being studied here were seemingly chimeric from the FISH studies shown in Chapter 5, this would also demonstrate whether, in fact, two separate YACs present within the same yeast clone were responsible for the results.) The gel in Figure 6.1, and another loaded with identical samples in the same lane order, were treated and blotted onto nylon. After cross-linking the DNA to the membranes, separate high stringency hybridizations with $^{32}$P-labelled full-length cDNA probes for FMO1, FMO2, FMO3, FMO4 and FMO5 were performed. On each occasion, $^{32}$P-labelled concatameric λ DNA pulse markers (identical to those loaded on the pulse field gel) were included in the probe mixtures to facilitate YAC size determination on the X-ray film following autoradiography. Each of these hybridizations will now be considered in turn.

6.3i Hybridization with FMO1
Probe hybridization was strongly centered on a band from 4X135-B12 (Figure 6.2, lanes d,e and f) in the same position as the postulated YAC band in Figure 6.1 (lanes d, e and f). The insert of the 4X135-B12 YAC has been shown to contain FMO1, so this was an expected result.

6.3ii Hybridization with FMO2
FMO2 probe produced an identical pattern of hybridization to FMO1, (Figure 6.2), confirming that 4X135-B12 also contains FMO2.

6.3iii Hybridization with FMO3
Once more, the probe hybridizes exclusively to YAC 4X135-B12 (ie the X-ray film produced by autoradiography appears nearly identical to Figure 6.2), so the FMO3 gene is also present within the insert of this clone.

6.3iv Hybridization with FMO4
FMO4 probe hybridized to YAC 4X135-B12 alone to give identical results to those obtained by FMO1, FMO2 and FMO3 probes, confirming that this YAC contains the genes for human FMO1, FMO2, FMO3 and FMO4 within its insert.
Figure 6.2. Southern hybridization analysis of yeast artificial chromosomes separated by pulse field gel electrophoresis: Probing with human FMO1. Shown here is an X-ray film following autoradiography of a Southern blot of a pulse field gel. Separated on the gel were genomic DNA preparations from yeast clones bearing yeast artificial chromosomes (YACs). The blot had previously undergone high-stringency hybridization with ³²P-labelled full-length human FMO1 cDNA and ³²P-labelled λ DNA concatemers (Sigma Pulse Marker). The FMO1 probe has hybridized strongly to the YAC genomic insert of clone 4X135-B12. Also visible are the λ DNA concatamer pulse markers originally loaded on the gel. Lane order as follows: Lanes a and t, λ Pulse Markers (Sigma) consisting of λ DNA concatemers (1-21) to give fragments ranging from 48.5-1019kb; Lanes b and s, whole intact yeast chromosomes from S. cerevisiae strain YPH80 (Sigma), sixteen chromosomes in total ranging in size from 225-2200kb; lanes d, e and f, YAC clone 4X135-B12, previously shown to have a non-chimeric insert containing FMO1, FMO2, FMO3 and FMO4; lanes h, i and j, YAC clone 4X78-F11, shown previously to bear a non-chimeric insert containing FMO5; lanes l, m and n, YAC clone 4X74-G7, also shown to have a non-chimeric insert bearing FMO5; lanes p, q and r, YAC clone 3008, bearer of a human genomic insert without FMO genes; lanes c, g, k and o are empty.
6.3v Hybridization with \textit{FMO5}

\textit{FMO5} probe hybridized to a single band from the 4X78-F11 (Figure 6.3, lanes h, i and j) and 4X74-G7 (Figure 6.3, lanes l, m and n) clone separations, but not the 4X135-B12 YAC band (Figure 6.3, lanes d, e and f). We can assume the bands for 4X78-F11 and 4X74-G7 signify a YAC in each case that has within its human genomic insert the \textit{FMO5} gene. As we have already shown that \textit{FMO5} exists within the inserts of the 4X78-F11 and 4X74-G7 YACs, but not that of 4X135-B12, this is an expected outcome. However, in Figure 6.1 it was noted that the YAC band could not be clearly observed for either 4X78-F11 or 4X74-G7 on the pulse field gel, so it was assumed that both were probably obscured by native chromosomes of a similar size. In the case of 4X78-F11, it is difficult to estimate the size of the YAC with any degree of accuracy, as its very large size means it has migrated to a region of the gel where the size-migration distance relationship no longer applies to any meaningful degree. Nevertheless, it would seem safe to assume that it is at least 1mb in size and was obscured on the photograph of the pulse field gel by the largest of the native chromosomes. 4X74-G7, on the other hand, is not so large and easier to estimate in size. Careful analysis of Figures 6.1 and 6.3 reveal that this YAC is about 550kb; hence if one examines lanes l, m and n of Figure 6.1, it is just possible to perceive the YAC as having migrated with native chromosomes 8 and 5 (which themselves migrated as a doublet on this gel, so here we actually have a triplet band).

6.4 Summary

Through the use of PFGE and PFGE followed by Southern hybridization, I have been able to determine the approximate sizes of three YAC inserts previously shown to contain \textit{FMO} genes. I have also shown that the yeast clones carrying these YACs contain no other YACs, a phenomenon that sometimes occurs during the construction of YAC libraries. Table 6.1 summarizes the data obtained so far in the characterization of these three YACs.
Figure 6.3. Southern hybridization analysis of yeast artificial chromosomes separated by pulse field gel electrophoresis: Probing with human FMO5. A Southern blot of a pulse field gel, on which had been separated genomic DNA preparations from yeast clones bearing yeast artificial chromosomes (YACs), was probed with ^3P-labelled full-length human FMO5 cDNA and ^3P-labelled lambda DNA concatemers (Sigma Pulse Marker) using high stringency hybridization conditions. Autoradiography produced the X-ray film shown above. The FMO5 probe has hybridized to the YAC genomic insert of clones 4X78-F11 and 4X74-G7. Also visible are the lambda DNA concatemer pulse markers originally loaded on the gel. Lane order as follows: Lanes a and t, lambda Pulse Markers (Sigma) consisting of lambda DNA concatemers (1-21) to give fragments ranging from 48.5-1019kb; Lanes b and s, whole intact yeast chromosomes from S. cerevisiae strain YPH80 (Sigma), sixteen chromosomes in total ranging in size from 225-2200kb; lanes d, e and f, YAC clone 4X135-B12, previously shown to have a non-chimeric insert containing FMO1, FMO2, FMO3 and FMO4; lanes h, i and j, YAC clone 4X78-F11, shown previously to bear a non-chimeric insert containing FMO5; lanes l, m and n, YAC clone 4X74-G7, also shown to have a non-chimeric insert bearing FMO5; lanes p, q and r, YAC clone 3008, bearer of a human genomic insert without FMO genes; lanes c, g, k and o are empty.
<table>
<thead>
<tr>
<th>YAC</th>
<th><em>FMO</em> gene present</th>
<th>Chromosome location</th>
<th>Chimeric/(&gt;1)YAC</th>
<th>Approximate insert size</th>
</tr>
</thead>
<tbody>
<tr>
<td>4X78-F11</td>
<td><em>FMO5</em></td>
<td>1q21</td>
<td>No</td>
<td>(\geq1000)kb</td>
</tr>
<tr>
<td>4X74-G7</td>
<td><em>FMO5</em></td>
<td>1q21</td>
<td>No</td>
<td>550kb</td>
</tr>
<tr>
<td>4X135-B12</td>
<td><em>FMO1</em>, <em>FMO2</em>, <em>FMO3</em> and <em>FMO4</em></td>
<td>1q23-24</td>
<td>No</td>
<td>480kb</td>
</tr>
</tbody>
</table>

Table 6.1. Characterization of three YAC clones isolated from a human genomic library. Determination of *FMO* gene content was by Southern hybridization analysis. The chromosome location from which the insert was originally derived was ascertained by fluorescence *in-situ* hybridization (FISH) to human metaphase chromosomes. Chimerism or the presence of more than one YAC was ruled out by the FISH analysis, pulsed field gel electrophoresis (PFGE) and PFGE followed by Southern hybridization. Approximate insert size was determined by PFGE alone or PFGE followed by Southern hybridization.
Chapter 7

Screening of a human chromosome 1-specific cosmid library for \textit{FMO} genes
7.1 Background

When setting out to obtain human genomic sequences of interest, a decision must be made as to what form of library will be screened. Given the size of the human genome, vectors capable of carrying inserts of sufficient size are required if the library is to be of a manageable size. Today, there is an ever-increasing degree of choice and availability in the matter, due mainly to the ongoing worldwide collaborative effort to sequence the entire human genome. Fully characterized libraries of various kinds are available without cost, the only requirement being that data obtained from such libraries be submitted as soon as is possible. Most available human genomic DNA libraries are still those that were constructed using yeast artificial chromosomes (YACs) and Chapter 4 (Isolation of YACs bearing FMO-containing human genomic inserts) details the outcome of screening such a library. YACs have the considerable advantage of carrying insert sizes unmatched by other cloning systems, up to 1Mb in size, rendering the screening of large, complex genomes quite feasible. However, disadvantages include frequent chimerism and the problems associated with handling, manipulating and characterizing such large DNA fragments, especially when the sequence of interest may be only a tiny fraction of the total insert. Other libraries have been constructed using, for example, bacteriophage P1 and, more recently, bacterial artificial chromosomes (BACs).

More specialized human genomic libraries include those that comprise only one of the chromosomes. Such chromosome-specific libraries are made possible through the use of flow cytometers. Specific chromosomes of interest can be stained with a dye, separated from other chromosomes on the basis of their characteristic staining pattern, size and shape, and then used to construct a library. A library of this kind has two principal advantages. Firstly, genes of interest may have been assigned to a particular chromosome by, for example, linkage analysis or PCR screening of somatic cell hybrids. If so, then screening a library specific for that chromosome renders the task of isolating that gene much easier. Secondly, as the total amount of DNA to be cloned is much reduced from the total genome, other vectors designed for carrying smaller inserts (for example, cosmids, which can take inserts up to 40kb in length) can be employed without the number of clones reaching astronomical proportions. Needless to say, smaller inserts are easier to handle, manipulate and characterize. However, they do considerably increase the risk of isolating only part of the gene of interest within a given clone, which in turn generates the necessity to assemble contiguous clones.
7.2 Screening strategy

A human chromosome 1-specific library constructed using the cosmid Lawrist 4 as vector was requested from the ICRF Reference Library Database (for more details of this library and techniques referred to in this chapter, please see Chapter 2: Materials and Methods, Section 2.5). The aim was to screen this library for clones bearing \( FMO \) sequences. Cosmids thus isolated could then be used to assist in characterizing the putative \( FMO \) gene cluster on human chromosome 1q (see Chapter 3: Localization of human \( FMO \) genes to chromosome 1q). This strategy was executed in conjunction with the screening of a human genomic YAC library (see Chapter 4: Isolation of YACs bearing \( FMO \)-containing human genomic inserts) for YAC clones bearing inserts containing human \( FMO \) sequences. Combining these efforts and isolating both cosmid and YAC human \( FMO \) clones was considered advantageous: for example, labelled \( FMO \)-bearing cosmids could be aligned on \( FMO \)-bearing YACs to establish the gene order of any cluster.

According to the Reference Library Database, the human genomic DNA used to construct the ICRF chromosome 1-specific cosmid library was partially digested with the restriction enzyme MboI before ligation into the BamH1 restriction enzyme cutting site of Lawrist 4. Such a strategy results in the regeneration of the BamH1 restriction enzyme cutting sequence at the site of ligation between vector and insert on 25% of occasions. The host organism was DH5 alpha. In total, the library consisted of 9216 clones spotted in duplicate on a single nylon filter. Two duplicate filters were provided.

7.3 Library Screening

Filters were hybridized under high-stringency conditions with a probe mixture including \( 32P \)-labelled full-length human \( FMO1, FMO2, FMO3, FMO4 \) and \( FMO5 \) cDNAs. Also included in the hybridization mixture was \( 35S \)-labelled Lawrist vector for assistance in establishing the coordinates of those 'positive' clones apparently bearing \( FMO \) sequences. After autoradiography, examination of the X-ray film revealed such clones as dark spots, whereas lighter spots were the result of vector-only binding (Figure 7.1).
Figure 7.1. Screening of an ICRF human chromosome-1 specific cosmid library for the presence of FMO genes. Shown here is an X-ray film produced by autoradiography following probing of a nylon filter spotted with 18432 cosmid clones arranged as high density arrays. (The entire chromosome library actually consists of 9216 clones, each of which has been spotted twice on the filter.) High-stringency hybridization took place with $^{32}$P-labelled human FMO1, FMO2, FMO3, FMO4 and FMO5 full-length cDNAs as probes. Also included was $^{35}$SdATP-labelled Lawrist (cosmid) vector. Hybridization of FMO probe to a clone on the filter produces a dark spot: in this case, twelve such spots were identified for selection. Establishing the coordinates of these spots was aided by the appearance of fainter spots, a result of hybridisation by the Lawrist vector to other clones.
In all, twelve clones were considered to have strongly hybridized to the FMO probe mixture. The coordinates of these clones were established and requested from the Reference Library Database. Their full names are listed in Table 7.1. Note: For convenience, from this point onwards in the text the clones will be named ‘1-12’, respectively, as listed in Table 7.1.

7.4 Verification of requested clones

Clones were received as stabs on agar. Normally, the next step might be to make glycerol stocks directly from these stabs. However, the Reference Library Database inform recipients of their clones that the microtiter plate wells from which they are taken do not necessarily contain a homogenous mixture of clones. As a result, the first step was to show that single colonies derived from these stabs contained constructs capable of hybridizing to the same probe mixture used in the initial filter screening.

To this end, the ‘replica plating’ method was used. Media inoculated from the stabs were incubated and the resultant cultures spread on agar plates (media and agar always contained a selective antibiotic to maintain the cosmid). Once colonies had grown on the plates, they were lifted onto nylon filter disks. Following treatment to expose resident nucleic acids and cross-link them to the nylon, these disks were probed with $^{32}$P-labelled full-length cDNA probes for FMO1, FMO2, FMO3, FMO4 and FMO5 using hybridization conditions of high stringency identical to those used for the initial screening of the library. Autoradiography produced X-ray film indicating which colonies had hybridized to the FMO probe mixture, as where bound probe existed there was exposure of the film (Figure 7.2). It was possible to then return to the agar plates, identify these ‘positive’ colonies and pick them to make glycerol stocks. Examination of the X-ray films for all twelve clones revealed that there was a considerable variation in the ratio of colonies grown on the agar plate to those colonies subsequently shown to hybridize to the FMO probe mixture. In other words, the apparent homogeneity of some of the stabs originally sent was less than 100% with respect to FMO hybridizing capability (Table 7.2). In fact, only four of the stabs, those for clone numbers 2, 6, 7 and 11, appeared homogenous in this respect. The stab for clone number 3 gave colonies that in every case failed to show any FMO binding affinity.
<table>
<thead>
<tr>
<th>Clone number</th>
<th>Cosmid (Lawrist 4) clone name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ICRFcl112H22104QD4</td>
</tr>
<tr>
<td>2</td>
<td>ICRFcl112I1678QD4</td>
</tr>
<tr>
<td>3</td>
<td>ICRFcl112J1872QD4</td>
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<td>8</td>
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<td>ICRFcl112C0396QD4</td>
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</tr>
<tr>
<td>12</td>
<td>ICRFcl112F1167QD4</td>
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</tbody>
</table>

Table 7.1. Names of twelve cosmid clones suspected of bearing inserts with FMO-like sequences. Clones were selected from the ICRF human chromosome 1-specific cosmid library following high-stringency hybridization to a probe mixture containing full-length human FMO1, FMO2, FMO3, FMO and FMO5 cDNAs.
Figure 7.2. Identifying and selecting DH5 alpha colonies containing cosmid constructs bearing inserts with human FMO-like sequences. Twelve cosmid clones had previously been selected from the ICRF human chromosome 1-specific library on the basis of their ability to hybridize to a probe mixture containing $^{32}$P-labelled full-length cDNAs for human FMO1, FMO2, FMO3, FMO4 and FMO5. Single colonies derived from stabs representing these clones were grown on agar plates and lifted onto nylon filter disks. Following treatment to expose resident nucleic acids, the filters were hybridized to the same labelled FMO probe mixture. Autoradiography produced X-ray films on which colonies capable of hybridizing to the labelled FMO DNAs were visible as dark spots. Using the film, it was possible to return to the agar plate and pick these colonies. Glycerol stocks made from these colonies could thus be safely assumed homogenous and potentially bearing of human FMO gene sequences. Shown here is the X-ray film autoradiograph for cosmid clone ICRFcl12C1795QD4. Numerous FMO-binding colonies can be seen, of which four were picked, grown and stored as glycerol stocks.
Table 7.2. Analysis of twelve stabs consisting of cosmid clones potentially bearing human FMO sequences by replica plating and subsequent hybridization to FMO probe. Twelve cosmid clones had previously been selected from the ICRF human chromosome 1-specific cosmid library on the basis of their apparent ability to hybridize to an FMO probe mixture (32P-labelled full-length human FM01, FM02, FM03, FM04 and FM05 cDNAs). Clones were received as stabs and rechecked for their FMO-binding capability as well as their homogeneity via the replica plating method. Shown here, for each of the clones, is the percentage of colonies grown on agar plates that were subsequently shown, after transfer to nylon disks, to hybridize to the same FMO probe mixture. Hence, 100% would indicate a stab of homogenous clone content with respect to FMO-binding capability, and so forth. Also shown is a subjective estimate of the relative degree of FMO probe hybridization to colony DNA under high-stringency conditions, based on visual examination of X-ray film exposure i.e. 'strong' refers to very dark spots on the film corresponding to colony positions on the agar plate, and so on. Such comparisons are valid as probe and all materials and methods were identical for all disks.

<table>
<thead>
<tr>
<th>Clone number</th>
<th>Cosmid (Lawrist4) clone name</th>
<th>Approx. % colonies bound to FMO probe</th>
<th>Relative probe hybridization intensity</th>
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</thead>
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<tr>
<td>1</td>
<td>ICRFc112H22104QD4</td>
<td>10</td>
<td>Medium</td>
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<td>ICRFc112I1678QD4</td>
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</tr>
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Furthermore, there appeared to be significant variation in the relative intensity of probe hybridization between colonies from different plates, indicating that the respective cosmid clones probably bear inserts with varying degrees of homology to the human *FMO* sequences in the probe mixture, or that the length of complementary sequence between probe and insert is quite small in some cases (Table 7.2). Such comparison of relative X-ray film exposures by eye, although clearly subjective, is worthwhile because probe (aliquots from the same single labelling reaction were used in all cases) hybridization conditions (hybridization solution, hybridization temperature, filter-wash solutions, filter-wash temperature and number of filter washes) and autoradiography conditions (type of film, length of exposure, temperature of exposure etc.) were matched for all filters. The four seemingly homogenous *FMO*-binding clones appeared to hybridize most strongly to the *FMO* probe mixture, producing very intense areas of exposure on the X-ray film in those areas corresponding to colonies on the agar plate. Clones 4, 5, 8, 9, 10 and 12 all appeared to hybridize very weakly to the probe. In the case of clone number 1, those colonies binding the probe produced an exposure of apparent intensity somewhere between these two extremes.

As a precaution, numerous ‘positive’ colonies were picked and grown to make glycerol stocks. These were labelled according to the assigned number (1-12 bar 3) of the original clone stab followed by the colony number in picking order. For example, in the case of the agar plate on which a culture grown from the stab representing clone ICRFcl112H22104QD4 (assigned clone 1) was spread, the second colony picked from that plate is labelled as ‘clone 1, colony 1.2’ or simply ‘colony 1.2’.

### 7.5 Southern blotting studies

Cultures were grown from glycerol stocks for each of the clones except number 3 and cell pellets produced, from which purified cosmid DNA was isolated and purified using commercial kits. The DNA concentration of the cosmid preparations was measured spectrophotometrically ($A_{260}$). Samples of each of the eleven cosmid DNA preparations were digested with the restriction enzyme BamH1. It must be remembered that BamH1 would be expected to completely liberate the human genomic DNA from Lawrist 4 on only 12.5% of occasions, as the vector-insert sticky end ligation reactions took place between BamH1 and Mbo1 restriction enzyme cutting sequences, respectively. Having established successful digestion of the cosmid DNA* by agarose gel electrophoresis, digests
were loaded on a 0.7% agarose gel and separated (Figure 7.3) before being subject to the Southern blotting protocol. Numerous Southern blots were carried out in this way, so generating the many nylon membranes required to further characterize the cosmid clones with regard to possible FMO content.

*Examination of Figure 7.3 reveals that many of the clones show faint bands in the gel and that some very high molecular weight fragments remain. The most likely explanation of this would be that, despite strenuous efforts, digestion with BamH1 was not 100% complete in the case of some of the clones.

**7.6 Probing with all five human FMO sequences simultaneously**

One of the Southern blot membranes was incubated with the same FMO probe mixture used to date (32P-labelled full-length cDNAs for FMO1, FMO2, FMO3, FMO4 and FMO5), again using high stringency hybridization conditions. Autoradiography produced an X-ray film showing that the probe sequences had hybridized very strongly to the cosmid DNA fragments of colonies picked from clone numbers 2, 6, 7 and 11 (Figure 7.4). Significant hybridization appears to have occurred in the case of clone 1, albeit at much reduced levels. Indeed, fragments became clearly evident on the film after exposure times of less than 1 hour, with background non-specific hybridization completely absent. When the time came to strip the membrane of bound probe, numerous washes with boiling H2O were required. Oddly however, in the case of clones 1 and 6, individual bands visible on the X-ray film (and only very faintly visible in Figure 7.3) can only be seen for one of the two colonies separated on the gel, even though these two colonies produced cosmid preparations with identical 'fingerprints' in all other respects. In the case of clone 1, this band is produced only from colony 1.1 and is the only fragment that appears to bind significant amounts of the probe. For clone 6, the extra band appears only in the case of colony 6.1. It is worthwhile comparing the FMO-binding bands on this film with the bands evident on the ethidium bromide-stained agarose gel on which the BamH1 digests of these four clones were separated (Figure 7.3 shows the gel that was blotted to produce the nylon membrane subjected to autoradiography to give Figure 7.4). We can see that in each case almost every fragment produced has hybridized to the probe mixture. This is a conceivable outcome if one remembers that the human genomic sequences of the inserts may well remain attached to the cosmid vector sequences, and that the inserts themselves are only up to 40kb in size. Overall, the data supports the findings of the replica plating experiments (Table 7.2).
Figure 7.3. Separation of cosmid DNA preparations suspected of bearing human FMO inserts by agarose gel electrophoresis prior to Southern transfer. Shown here is just one of numerous 0.7% agarose gels on which cosmid DNA preparations from individual clones were separated prior to Southern transfer following digestion with HindIII. This generated the numerous blots required to probe for the presence of individual FMO genes within these clones. The eleven clones originate from stabs requested from the Reference Library Database on the basis of their ability to hybridize to an FMO probe mixture (radiolabelled full length FMO1, FMO2, FMO3, FMO4 and FMO5 cDNAs) during the screening of an ICRF human chromosome 1 cosmid library. Each clone had subsequently been shown to bind the same FMO probe mixture to varying degrees following replica plating and colony hybridization. The names of these eleven clones are given here as ‘1-12’ for convenience sake (clone number 3 is absent as it displayed no binding affinity for the FMO probe mixture); the actual names to which these numbers refer can be found in Table 1. In some cases, cosmid DNA preparations from two separate FMO-binding colonies grown on the same plate (i.e. originating from the same stab) have been run adjacently on the gel. Where this is so, the colonies picked are named as, for example, 1.1, 1.2 and so forth. Lane order in this case was as follows: a and t, ^35SodATP-radiolabelled Gibco-BRL 1-kb molecular weight standards; b, cosmid clone 1, colony 1.1; c, cosmid clone 1, colony 1.2; d, cosmid clone 2, colony 2.2; e, cosmid clone 2, colony 2.3; f, cosmid clone 4, colony 4.1; g, cosmid clone 5, colony 5.1; h, cosmid clone 5, colony 5.2; i, cosmid clone 6, colony 6.1; j, cosmid clone 6, colony 6.2; k, cosmid clone 7, colony 7.1; l, cosmid clone 8, colony 8.1; m, cosmid clone 8, colony 8.2; n, cosmid clone 9, colony 9.1; o, cosmid clone 9, colony 9.2; p, cosmid clone 10, colony 10.1; q, cosmid clone 11, colony 11.1; r, cosmid clone 12, colony 12.1, and; s, human genomic DNA cut by the restriction enzyme BamH1 (to possibly show corresponding FMO-hybridizing bands). Note: in some cases it would appear that digestion with HindIII has not followed to 100% completion, judging by the presence of certain faint bands and very high molecular weight fragments.
Figure 7.4. Use of Southern blotting to reveal human FMO sequences within DNA fragments produced by BamH1 digestion of cosmid clones. Shown above is an X-ray film generated by autoradiography of a Southern blot. Blot consisted of cosmid clones cut by the restriction enzyme BamH1 that had been probed with a mixture of 32P-labelled full-length cDNAs for human FMO1, FMO2, FMO3, FMO4 and FMO5 using high stringency hybridization conditions. Cosmid clones were derived from a human chromosome 1-specific library and were suspected, at least in some cases, of containing sequences within their inserts for one or more of the FMO genes. Lane order as follows (see Table 1 and Figure 3 legend for an explanation of the labelling system used for clones in this thesis): lane a, 35SdATP-labelled molecular weight standards (Gibco-BRL 1kb ladder); lane b, cosmid clone 1, colony 1.1; lane c, cosmid clone 1, colony 1.2; lane d, cosmid clone 2, colony 2.2; lane e, cosmid clone 2, colony 2.3; lane f, cosmid clone 4, colony 4.1; lane g, cosmid clone 5, colony 5.1; lane h, cosmid clone 5, colony 5.2; lane i, cosmid clone 6, colony 6.1; lane j, cosmid clone 6, colony 6.2; lane k, cosmid clone 7, colony 7.1; lane l, cosmid clone 8, colony 8.1; lane m, cosmid clone 8, colony 8.2; lane n, cosmid clone 9, colony 9.1; lane o, cosmid clone 9, colony 9.2; lane p, cosmid clone 10, colony 10.1; lane q, cosmid clone 11, colony 11.1; lane r, cosmid clone 12, colony 12.1; lane s, human genomic DNA cut by the restriction enzyme BamH1 (to possibly show corresponding FMO-hybridizing bands), and; lane t, 35SdATP-labelled molecular weight standards (Gibco-BRL 1kb ladder). Exposure time for this film was just 90min, indicating the specificity of the FMO probe for sequences within the inserts of cosmid clones 2, 6, 7, 11 and, to a lesser extent, 1. It appears that the probe has bound to every fragment produced by HindIII digestion of the 2, 6, 7 and 11 clones (comparing Figure 3). Somewhat anomalous is the extra band apparent for colony 6.1 compared to 6.2 and also colony 1.1 compared to 1.2 (both faintly visible upon close inspection of Figure 3), when the respective colony cosmid DNA preparations for these two clones produce identical ‘fingerprints’ in all other respects.
At this point, then, it seemed reasonable to assume that four, possibly five, cosmid clones had been isolated bearing human genomic inserts from the regions of chromosome 1 coding for the known \textit{FMO} genes. The next step involved probing the blots with each \textit{FMO} cDNA in isolation to ascertain which \textit{FMO} genes, if any, were present within the clones.

\section*{7.7 Probing with human \textit{FMO1} alone}

One of the Southern blots was incubated with $^{32}$P-labelled full-length human \textit{FMO1} cDNA using high stringency hybridization conditions. Autoradiography produced the X-ray film shown in Figure 7.5. In Figure 7.6, the agarose gel that was blotted to produce the nylon membrane probed in this case is shown, for comparison purposes. As can be seen, the clones that have bound strongly to \textit{FMO1} sequence are numbers 2, 6, 7 and 11, the same clones that hybridized strongly to the \textit{FMO} probe mixture (Figure 7.4). A much weaker level of hybridization can be seen upon close inspection for clones 1 (only colony 1.1 once more) and 12, in the form of single faint bands in each case. Unexpectedly perhaps, the pattern of bands that have appeared on the X-ray films shown in Figures 7.3 and 7.4 are very similar. Taken in isolation, the most likely explanation for such a finding would be that the clones probably contain just \textit{FMO1}. However, the following results from probing with the other \textit{FMO} family members in isolation would seem to refute this.

\section*{7.8 Probing with human \textit{FMO2} alone}

$^{32}$P-labelled full-length human \textit{FMO2} cDNA was used to probe a blot using hybridization conditions of high stringency. Probe hybridization to cosmid clone DNA fragments was so strong in some cases that the nylon membrane needed extra 65°C washes with low salt solution (0.1xSSPE, 1% SDS) to reduce the signal. Figure 7.7 shows the X-ray film produced by autoradiography following these extra washes. The probe appears to have bound very strongly to all the sequences from the cosmid clones numbered 2, 6, 7 and 11 (including the additional fragment of colony 6.1 noted in Figure 7.4). A very much lower level of hybridization is seen for the other clones, with clone number 1 being the possible exception as it appears to have bound significant amounts of the probe on one of its fragments (the same one as was first noted in Figure 7.4) in the case of colony 1.1 only.
Figure 7.5. Use of Southern blotting to reveal human *FMO1* sequences within DNA fragments produced by BamHI digestion of cosmid clones. Shown here is an X-ray film generated by autoradiography of a Southern blot consisting of cosmid clones cut by the restriction enzyme BamH1. The blot had been probed with ³²P-labelled full-length cDNA for human *FMO1* under hybridization conditions of high stringency. Cosmid clones were derived from a human chromosome 1-specific library and were suspected, at least in some cases, of containing sequences within their inserts for one or more of the *FMO* genes. Lane order as follows (see Table 1 and Figure 3 legend for an explanation of the labelling system used for clones in this thesis): lane a, ³²P-ATP-labelled molecular weight standards (Gibco-BRL 1kb ladder); lane b, human genomic DNA cut by the restriction enzyme BamH1 (to possibly show corresponding *FMO*-hybridizing bands); lane c, cosmid clone 1, colony 1.1; lane d, cosmid clone 1, colony 1.2; lane e, cosmid clone 2, colony 2.2; lane f, cosmid clone 2, colony 2.3; lane g, cosmid clone 4, colony 4.1; lane h, cosmid clone 5, colony 5.1; lane i, cosmid clone 5, colony 5.2; lane j, cosmid clone 6, colony 6.1; lane k, cosmid clone 6, colony 6.2; lane l, cosmid clone 7, colony 7.1; lane m, cosmid clone 8, colony 8.1; lane n, cosmid clone 8, colony 8.2; lane o, cosmid clone 9, colony 9.1; lane p, cosmid clone 9, colony 9.2; lane q, cosmid clone 10, colony 10.1; lane r, cosmid clone 11, colony 11.1; lane s, cosmid clone 12, colony 12.1, and; lane t, ³²P-ATP-labelled molecular weight standards (Gibco-BRL 1kb ladder). Exposure time for this film was 12hr. The *FMO1* probe appears to have hybridized with high specificity to sequences within the inserts of cosmid clones 2, 6, 7 and 11. It appears that the probe has bound to virtually every fragment produced by HindIII digestion of these clones (comparing Figure 6.6).
Figure 7.6. Separation by agarose gel electrophoresis of cosmid DNA preparations digested with HindIII prior to Southern transfer. Shown here is a 0.7% agarose gel on which HindIII-cut cosmid DNA preparations from individual clones were separated prior to Southern transfer. The blot produced was used in Figure 5. The eleven clones originate from stabs requested from the Reference Library Database on the basis of their seeming ability to hybridize to an FMO probe mixture (radiolabelled full length FMO1, FMO2, FMO3, FMO4 and FMO5 cDNAs) during the screening of an ICRF human chromosome 1 cosmid library and each clone had subsequently been shown to bind the same FMO probe mixture to varying degrees following replica plating and colony hybridization. The names of these eleven clones are given here as ‘1-12’ (clone number 3 displayed no binding affinity for the FMO probe mixture after replica plating and so was excluded); the actual names to which these numbers refer can be found in Table 1. Sometimes, cosmid DNA preparations from two separate FMO-binding colonies but originating from the same stab have been run adjacently on the gel. Where this is so, the colonies picked are named as, for example, 1.1, 1.2 and so forth. Lane order in this case was as follows: a and t, 35S\textsuperscript{3}\textsuperscript{3}P\textsuperscript{3}dATP-radiolabelled Gibco-BRL 1-kb molecular weight standards; b, human genomic DNA cut by the restriction enzyme BamHI (to possibly show corresponding FMO-hybridizing bands); c, cosmid clone 1, colony 1.1; d, cosmid clone 1, colony 1.2; e, cosmid clone 2, colony 2.2; f, cosmid clone 2, colony 2.3; g, cosmid clone 4, colony 4.1; h, cosmid clone 5, colony 5.1; i, cosmid clone 5, colony 5.2; j, cosmid clone 6, colony 6.1; k, cosmid clone 6, colony 6.2; l, cosmid clone 7, colony 7.1; m, cosmid clone 8, colony 8.1; n, cosmid clone 8, colony 8.2; o, cosmid clone 9, colony 9.1; p, cosmid clone 9, colony 9.2; q, cosmid clone 10, colony 10.1; r, cosmid clone 11, colony 11.1, and; s, cosmid clone 12, colony 12.1. Note: in some cases it would appear that digestion with HindIII has not followed to 100% completion, judging by the presence of certain faint bands and very high molecular weight fragments.
Figure 7.7. Use of Southern blotting to reveal human *FMO2* sequences within DNA fragments produced by BamH1 digestion of cosmid clones. Above is an X-ray film produced by autoradiography of a Southern blot consisting of cosmid clones cut by the restriction enzyme BamH1. ³²P-labelled full-length cDNA for human *FMO1* was used to probe the blot using high stringency hybridization conditions. Cosmid clones were from a human chromosome 1-specific library and were suspected, at least in some cases, of containing sequences within their inserts for one or more of the *FMO* genes. Lane order as follows (see Table 1 and Figure 3 legend for an explanation of the labelling system used for clones in this thesis): lane a, ³²PβdATP-labelled molecular weight standards (Gibco-BRL 1kb ladder); lane b, cosmid clone 1, colony 1.1; lane c, cosmid clone 1, colony 1.2; lane d, cosmid clone 2, colony 2.2; lane e, cosmid clone 2, colony 2.3; lane f, cosmid clone 4, colony 4.1; lane g, cosmid clone 5, colony 5.1; lane h, cosmid clone 5, colony 5.2; lane i, cosmid clone 6, colony 6.1; lane j, cosmid clone 6, colony 6.2; lane k, cosmid clone 7, colony 7.1; lane l, cosmid clone 8, colony 8.1; lane m, cosmid clone 8, colony 8.2; lane n, cosmid clone 9, colony 9.1; lane o, cosmid clone 9, colony 9.2; lane p, cosmid clone 10, colony 10.1; lane q, cosmid clone 11, colony 11.1; lane r, cosmid clone 12, colony 12.1; lane s, human genomic DNA cut by the restriction enzyme BamH1 (to possibly show corresponding *FMO*-hybridizing bands), and; lane t, ³²PβdATP-labelled molecular weight standards (Gibco-BRL 1kb ladder). Exposure time for this film was just 2.5hr and followed additional high temperature, low salt washes of the nylon membrane, indicating the hybridization affinity between probe and cosmid clone sequences.
The results seen here, and those that follow for the other *FMO* probes, were unexpected, as it would appear that although the *FMO* probes are consistently hybridizing strongly to complimentary sequences in clones 2, 6, 7 and 11 (and possibly 1), these sequences could not be those of separate *FMO* genes, given that the *FMO1* and *FMO2* probes, for example, anneal to numerous identical fragments. It should be pointed out at this stage that the cDNAs used to make all of the probes mentioned in this Chapter were from exactly the same preparations used in the screening of the YAC human genomic library (Chapter 4) and subsequent Southern blots, where it was shown that the probes were specific for their respective genes.

### 7.9 Probing with human *FMO3* alone

Probing a Southern blot with $^{32}$P-labelled full-length human *FMO3* cDNA revealed that the strongest hybridization under high-stringency conditions is to most, if not all, of the fragments from clones 2, 6, and 7 (an additional band is again present in the case of colony 6.1) (Figure 7.8). There also appears to be significant hybridization to clones 1 (once again, a single fragment produced from colony 1.1) and 11. Hybridization to other clones is not evident.

### 7.10 Probing with human *FMO4* alone

$^{32}$P-labelled full-length human *FMO4* cDNA probe was found to hybridize strongly to clones 2 and 7, and less strongly to clone 6 (Figure 7.9) when hybridization conditions of high stringency were used. When the film was scrutinized closely, much weaker probe hybridization was revealed for clones 11 and 1 (the single band of colony 1.1 already noted).

### 7.11 Probing with human *FMO5* alone

Probing with $^{32}$P-labelled full-length human *FMO5* cDNA produced very strong hybridization to the clones 2 and 7, with slightly less hybridization to clone 6 (Figure 7.10). Far weaker hybridization was evident for clones 1 (the same single band of colony 1.1) and 11.
Figure 7.8. Use of Southern blotting to reveal human \textit{FMO3} sequences within DNA fragments produced by \textit{BamH1} digestion of cosmid clones. Shown here is an X-ray film generated by autoradiography of a Southern blot consisting of cosmid clones cut by the restriction enzyme \textit{BamH1}. The blot was probed at high stringency with $^{32}$P-labelled full-length cDNA for human \textit{FMO3}. Cosmid clones were derived from a human chromosome 1-specific library and were suspected, at least in some cases, of containing sequences within their inserts for one or more of the \textit{FMO} genes. Lane order as follows (see Table 1 and Figure 3 legend for an explanation of the labelling system used for clones in this thesis): lane \textit{a}, $^{35}$S\textit{dATP}-labelled molecular weight standards (Gibco-BRL 1kb ladder); lane \textit{b}, human genomic DNA cut by the restriction enzyme \textit{BamH1} (to possibly show corresponding \textit{FMO}-hybridizing bands); lane \textit{c}, cosmid clone 1, colony 1.1; lane \textit{d}, cosmid clone 1, colony 1.2; lane \textit{e}, cosmid clone 2, colony 2.2; lane \textit{f}, cosmid clone 2, colony 2.3; lane \textit{g}, cosmid clone 4, colony 4.1; lane \textit{h}, cosmid clone 5, colony 5.1; lane \textit{i}, cosmid clone 5, colony 5.2; lane \textit{j}, cosmid clone 6, colony 6.1; lane \textit{k}, cosmid clone 6, colony 6.2; lane \textit{l}, cosmid clone 7, colony 7.1; lane \textit{m}, cosmid clone 8, colony 8.1; lane \textit{n}, cosmid clone 8, colony 8.2; lane \textit{o}, cosmid clone 9, colony 9.1; lane \textit{p}, cosmid clone 9, colony 9.2; lane \textit{q}, cosmid clone 10, colony 10.1; lane \textit{r}, cosmid clone 11, colony 11.1; lane \textit{s}, cosmid clone 12, colony 12.1; and; lane \textit{t}, $^{35}$S\textit{dATP}-labelled molecular weight standards (Gibco-BRL 1kb ladder). Exposure time for this film was 24hr. This was not long enough for the radiolabelled molecular weight standards to appear.
Figure 7.9. Use of Southern blotting to reveal human FMO4 sequences within DNA fragments produced by BamH1 digestion of cosmid clones. Above is an X-ray film generated by autoradiography of a Southern blot. The blot was of cosmid clones cut by the restriction enzyme BamH1 and had been probed at high stringency with $^{32}$P-labelled full-length human FMO4 cDNA. Cosmid clones were derived from a human chromosome 1-specific library and were suspected, at least in some cases, of containing sequences within their inserts for one or more of the FMO genes. Lane order as follows (see Table 1 and Figure 3 legend for an explanation of the labelling system used for clones in this thesis): lane a, $^{35}$S$\alpha$dATP-labelled molecular weight standards (Gibco-BRL 1kb ladder); lane b, human genomic DNA cut by the restriction enzyme BamH1 (to possibly show corresponding FMO-hybridizing bands); lane c, cosmid clone 1, colony 1.1; lane d, cosmid clone 1, colony 1.2; lane e, cosmid clone 2, colony 2.2; lane f, cosmid clone 2, colony 2.3; lane g, cosmid clone 4, colony 4.1; lane h, cosmid clone 5, colony 5.1; lane i, cosmid clone 5, colony 5.2; lane j, cosmid clone 6, colony 6.1; lane k, cosmid clone 6, colony 6.2; lane l, cosmid clone 7, colony 7.1; lane m, cosmid clone 8, colony 8.1; lane n, cosmid clone 8, colony 8.2; lane o, cosmid clone 9, colony 9.1; lane p, cosmid clone 9, colony 9.2; lane q, cosmid clone 10, colony 10.1; lane r, cosmid clone 11, colony 11.1; lane s, cosmid clone 12, colony 12.1; and; lane t, $^{35}$S$\alpha$dATP-labelled molecular weight standards (Gibco-BRL 1kb ladder). It would appear that the molecular weight standards are poorly labelled and so have not appeared on the film.
Figure 7.10. Use of Southern blotting to reveal human FMO5 sequences within DNA fragments produced by BamH1 digestion of cosmid clones. Autoradiography of a Southern blot, probed at high stringency with $^{32}$P-labelled full-length human FMO5 cDNA, generated the X-ray film shown above. Cross-linked on the blot were cosmid clones cut by the restriction enzyme BamH1. Cosmid clones were derived from a human chromosome 1-specific library and were suspected, at least in some cases, of containing sequences within their inserts for one or more of the FMO genes. Lane order as follows (see Table 1 and Figure 3 legend for an explanation of the labelling system used for clones in this thesis): lane a, $^{35}$S$d$ATP-labelled molecular weight standards (Gibco-BRL 1kb ladder); lane b, human genomic DNA cut by the restriction enzyme BamH1 (to possibly show corresponding FMO-hybridizing bands); lane c, cosmid clone 1, colony 1.1; lane d, cosmid clone 1, colony 1.2; lane e, cosmid clone 2, colony 2.2; lane f, cosmid clone 2, colony 2.3; lane g, cosmid clone 4, colony 4.1; lane h, cosmid clone 5, colony 5.1; lane i, cosmid clone 5, colony 5.2; lane j, cosmid clone 6, colony 6.1; lane k, cosmid clone 6, colony 6.2; lane l, cosmid clone 7, colony 7.1; lane m, cosmid clone 8, colony 8.1; lane n, cosmid clone 8, colony 8.2; lane o, cosmid clone 9, colony 9.1; lane p, cosmid clone 9, colony 9.2; lane q, cosmid clone 10, colony 10.1; lane r, cosmid clone 11, colony 11.1; lane s, cosmid clone 12, colony 12.1, and; lane t, $^{35}$S$d$ATP-labelled molecular weight standards (Gibco-BRL 1kb ladder). It would appear that the molecular weight standards are poorly labelled and so have appeared only very faintly on the film.
7.12 Summary

Screening a human chromosome-1 specific cosmid library with a probe mixture containing all five known human FMO cDNAs identified twelve cosmid clones for further characterization. Replica plating suggested eleven of these to be worthy of analysis for the presence of FMO sequences within their inserts via Southern hybridization analysis. The outcome of these studies are summarized in Table 7.3. It was found that cosmid clones given the abbreviated names 2 and 7 hybridized to each of the five human FMO cDNA probes to a very high degree in each case. Clone 6 was also shown to strongly bind all five FMO probes, but to a slightly reduced extent. Clone 11 bound FMO1 and FMO2 probes strongly and to a lesser extent the other FMO probes. One of the two colonies examined for clone 1 produced a single fragment that consistently displayed a low degree of hybridization affinity for each of the FMO probes. Finally, clone 12 produced a fragment that weakly bound FMO1 probe.

We can conclude that cosmid clones have been isolated from a human genomic library with inserts bearing a significant degree of homology to human FMO sequences. In the cases of clones 2, 6, 7 and 11 we can say that the degree of homology must be very high for a number of reasons. Firstly, the hybridization conditions used were of high stringency and were the same as those used to successfully screen the human genomic insert sequences of a YAC library for the presence of human FMO sequences (Chapter 4). Indeed, in the case of FMO2 hybridization, extra high-temperature, low-salt washes were required to lower the signal to a degree that made autoradiography practical. Secondly, hybridization is highly specific for the inserts of these clones, as evidenced by the absence of hybridization to other cosmid clones. Background hybridization to the membrane was also non-existent. Thirdly, the full-length FMO probes used were well characterized and verified to be genuine. They originated from the same sample preparation as was used for the successful screening of the aforementioned human genomic YAC library. Finally, such was the extent of hybridization that exposure times for the films undergoing autoradiography were for as little as 2.5 hours. Even in the case of clone 1, colony 1.1, where the degree of hybridization was relatively weak, it is likely that the insert contains a region of sequence with significant homology to all five FMO sequences.
Table 7.3. Relative degree of hybridization between cosmid clones and full-length human cDNA *FMO* probes. Clones listed above were those selected from the ICRF human chromosome 1-specific cosmid library following high-stringency hybridization to a probe mixture containing full-length human *FMO1*, *FMO2*, *FMO3*, *FMO4* and *FMO5* cDNAs. All bar number 3 were shown to some degree to anneal to the same probe mixture after replica plating, albeit to widely varying degrees (Table 2). Some of the apparent/possible *FMO*-binding colonies so identified had cosmid preparations made from them and these were cut with BamH1 before separation on an agarose gel and Southern transfer. Shown above is a crude and subjective estimate as to the relative degree to which radiolabelled individual full-length human *FMO* cDNA probes hybridized to the DNA fragments present on the membrane blot. This was undertaken by visual examination of the X-ray films produced by autoradiography (Figures 5, 7, 8, 9 and 10). The number of plus (+) symbols denotes the apparent relative affinity of the probe for the clone concerned, whereas a dash symbol (-) indicates that no specific binding to any significant degree was evident on the film. 'N/A' denotes 'not applicable'. *, denotes that for clone 1, probe binding occurred on just one fragment for only one of the two colonies subjected to the Southern hybridization analysis (colony 1.1).

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To isolate human genomic sequences from a chromosome 1-specific library with a high degree of homology to human FMO sequences would give grounds for considerable optimism that actual human genomic regions spanning these genes had been isolated, particularly in light of the fact that we now know all five human FMO genes to be located on the long arm of chromosome 1 (Chapter 3). Unfortunately, the Southern hybridization data would question such an assumption. First of all, in each case the clones that bind the FMO probes produce a fragment ‘fingerprint’ practically identical whichever of the five probes are used. In other words, individual fragments are strongly hybridizing to all five human FMO sequences (cross-hybridization between separate FMO sequences can be ruled out as the hybridization conditions used have previously been shown to prevent this, for example in the case of the YAC library screening already mentioned). Although we already know that FMO1, FMO2, FMO3 and FMO4 are all clustered at 1q23-24 (Chapter 5: Fluorescence in-situ hybridization analysis of YAC clones bearing human genomic sequences containing FMO genes), it is clearly an impossibility for the fragments produced by BamH1-cutting of the cosmid inserts, never mind the inserts themselves (40kb at most), to span all of these genes (shown to each be in the region of 20-50kb in size in Chapter 4). Second, data presented in Chapter 5 showed that FMO5 resides some distance from the other four FMO genes at 1q21 and hybridization with this sequence is as strong as for the other sequences.

However, two other explanations for the results, apart from the presence of known FMO genes within the clone inserts, must be considered. Firstly, it is perfectly possible that additional FMO genes are awaiting discovery (it has been reported that low-stringency hybridization analysis of genomic DNA suggests this to be the case (344)). If these additional genes were created by gene duplication at the same time as the other known FMO genes, then they would be expected to have sequence identities of just 52-57% with the probes used (31, 86) (see Section 1.3, Subsection 1.31: Primary structures of the mammalian FMOs and Table 1.1 in Chapter 1: An introduction to the enzymology and molecular biology of the flavin-containing monooxygenases and the scope of this thesis). Thus, while the presence of a ‘new’ FMO gene might explain the equal homology of all five FMO probes to the insert sequences, in the instance of clones 2, 6, 7 and 11 the level of hybridization indicates a degree of homology considerably in excess of this. Even clone 1, colony 1.1, with its single, weakly hybridizing fragment, probably contains sequence with a level of homology greater than this. We cannot rule out that further duplication events occurred following those that took place.
from the common ancestral gene, but these would produce genes with a greater than 51-57% homology to only one of the five known *FMO* gene sequences.

The other explanation to be considered is that of pseudogenes, regions of genomic sequence bearing homology to known gene sequences but which are incapable of producing a functional gene product. Pseudogenes are well documented in the case of gene families, a good example being the β-like globin gene cluster on human chromosome 11 which contains two pseudogenes among five functional genes, all of which are believed to have resulted from gene duplications (181). Duplication of genomic DNA bearing active genes has been postulated to occur by a number of ways, some of which do not lead to the generation of further active genes (for example, because the promoter region has not been duplicated along with the coding sequence). Such regions of apparently useless DNA will be the subject of ‘sequence drift’, the steady, uninterrupted and accumulated change of sequence over time. Even the precise duplication of all of the genetic components needed to produce another gene that can be actively transcribed and the mRNA translated to produce a functional protein (as occurred to produce the mammalian *FMO* gene family) may result in some or all of the newly acquired gene sequences rapidly becoming non-functional (through sequence drift) over the passage of generations if they fail to confer selective advantage to the host. Either way, whilst *FMO* pseudogenes, if in existence, would in theory be capable of hybridization to *FMO* probes, it is likely that the rapid decay of their sequence would have rendered them unable to do so under the high stringency hybridization conditions used in this case. Even if the pseudogene(s) had arisen relatively recently and significant sequence drift had not yet occurred, we would not expect hybridization to all five *FMO* probes with comparable affinity.

The identity of the inserts responsible for strongly hybridizing the *FMO* probe sequences, if not *FMO* genes (or pseudogenes) themselves, remains at this stage a mystery. If regions of the human genome capable of strongly binding five *FMO* sequences sharing only 51-57% homology and spanning only around 40kb in size do exist, it might seem strange that they were not more detected upon screening a YAC human genomic DNA library (*Chapter 4*), although all genomic libraries vary in their degree of representation of genomic regions. Nevertheless, it was considered worthwhile subjecting these clones to fluorescence *in-situ* hybridization (FISH) with human metaphase chromosomes to establish their respective chromosome locations. This is discussed in the
following Chapter (Chapter 8: Analysis of cosmid clones isolated from a chromosome 1-specific library by fluorescence in-situ hybridization).
Chapter 8

Analysis of cosmid clones isolated from a chromosome 1-specific library by fluorescence in-situ hybridization to human metaphase chromosomes
8.1 Background

In the previous Chapter (Chapter 7: Screening of a human chromosome 1-specific cosmid library for FMO genes), an account was given of how a number of cosmid clones were isolated from the ICRF human chromosome 1-specific library on the basis of an apparent ability to hybridize to an FMO probe mixture (full-length radiolabelled FM01, FM02, FM03, FM04 and FM05 cDNAs). Subsequent replica plating and Southern hybridization experiments revealed that four of the clones (clone numbers 2, 6, 7 and 11) were capable of consistently hybridizing to some or all of the five FMO gene sequences, to an extent that sequence homology between the human genomic inserts of these clones and FMO sequences were in all probability very high. Unfortunately however, the ability of individual fragments produced by BamH1 digestion of these clones to hybridize to more than one or all of the five FMO gene sequences seemed to rule out that the inserts of these clones contained actual FMO genes. An additional clone (clone number 1, colony 1.1) displayed a lower level of hybridization affinity for all five FMO gene probes on just a single fragment produced by BamH1 digestion.

It was deemed worthwhile characterizing these clones further by determining the genomic location of their inserts. This was undertaken by performing fluorescence in-situ hybridization (FISH) to human metaphase chromosomes using the clones as probes. In Chapter 5 (Fluorescence in-situ hybridization analysis of YAC clones bearing human genomic sequences containing FMO genes), I described how this technique was used to determine the genomic location of yeast artificial chromosome (YAC) clone inserts strongly suspected of containing FMO gene sequences. From this, it was shown that the FM01, FM02, FM03 and FM04 genes reside at 1q23-24 and the FM05 gene at 1q21. Once again, probes (cosmid clone DNA preparations in this case) were fluorescently labelled and hybridized to treated human metaphase chromosomes (for methodological details, please see Chapter 2: Materials and Methods, Section 2.6). The location of the hybridization signal (or signals if the clone insert is chimeric i.e. is the result of coligation events between fragments from different parts of the genome) was then determined and given in terms of the corresponding G (Giemsa)-band.

The cosmid clones chosen to undergo FISH analysis were the five mentioned above, namely clone 1, colony 1.1, clone 2, colony 2.2, clone 6, colony 6.1, clone 7, colony 7.1 and clone 11, colony 11.1. The genomic locations thus identified are listed in turn for each of the clones below.
8.2 Cosmid clone 1, colony 1.1

A single region of hybridization is visible at 15q11-13 (Figure 8.1). Thus, the non-chimeric insert of this clone does not originate from chromosome 1, even though the library is supposed to be chromosome 1-specific. This confirms earlier suspicions from the Southern hybridization data that the insert of this clone could not contain known FMO sequences. However, it was reported by Chen et al in 1993 that an individual afflicted with Prader-Willi syndrome, a complex multisystem disorder, was also a victim of fish odour syndrome or trimethylaminuria (264). Trimethylaminuria and its relation to the FMO enzymes is covered in some depth in Chapter 1 (An Introduction to the enzymology and molecular biology of the flavin-containing monooxygenases and the scope of this thesis), Section 1.11, Subsection 1.11i (Fish-odour syndrome/Trimethylaminuria). In brief, the disorder is characterized by an impaired capacity for metabolizing trimethylamine (TMA), a molecule released through the action of intestinal bacteria on common foodstuffs containing, for example, TMA N-oxide, choline, lecithin and carnitine. TMA is the highly odorous and volatile agent responsible for the smell of rotting fish, whereas its N-oxide is non-odorous. TMA taken up from the intestinal tract is converted, probably mainly in the liver, to the N-oxide, a reaction almost certainly catalyzed mainly by one or more of the FMO enzymes and subject to genetic polymorphism (257, 262, 266, 275, 279, 281, 288). Individuals unable to metabolize TMA in this manner excrete it in their breath, sweat and urine and so suffer from the odour of rotting fish, with dire psycho-social consequences. It follows that the likely genetic event responsible for trimethylaminuria is a mutation in the FMO gene coding for the enzyme primarily responsible for TMA N-oxidation, leading to a loss of activity of the enzyme. Prader-Willi syndrome has previously been associated with a deletion in the region 15q11-13 (348), and this was the case for the individual reported by Chen et al. Consequently, the authors speculate whether coincidentally the gene for the FMO enzyme primarily responsible for TMA N-oxidation is located within this deleted region, although they concede in their paper that the probability of this was lessened by the mapping of FMO1 and FMO4 (then labelled FMO2) to 1q.
Figure 8.1. FISH analysis to human metaphase chromosomes with cosmid clone 1, colony 1.1 as probe. Cosmid clone 1 was isolated from the ICRF human chromosome 1-specific library on the basis of an apparent hybridization affinity to an FMO probe mixture (radiolabelled FMO1, FMO2, FMO3, FMO4 and FMO5 full-length cDNAs). Colonies shown to bind the same probe mixture upon subsequent replica plating were picked, one of which was colony 1.1. Southern hybridization analysis revealed that cosmid DNA from this clone produced a single fragment upon digestion with BamH1 capable of low-level hybridization to all five FMO gene sequences individually. Above we show the results of fluorescence in-situ hybridization to human metaphase chromosomes with labelled clone as probe to reveal the genomic origin of the insert. Regions to which the clone hybridizes appear in green on the red chromosomes. A single location of hybridization is evident at 15q11-13 (arrowed).
If a hitherto undiscovered *FMO* gene does exist in the region 15q11-13, then a part of it may lie within the insert of this clone. The fact that hybridization of the five *FMO* probes was relatively poor yet comparable in each case to the one visible fragment on the film produced by BamH1 digestion of the clone might at first seem to support such a supposition, assuming this gene shares the typical 51-57% sequence identity shown between members of this gene family. However, the high stringency conditions used for the Southern hybridization experiments in this case were previously been shown to preclude cross-hybridization between *FMO* family members, so if this was a new *FMO* gene it would seem to be untypically homologous in its sequence to the other family members. To discover a hitherto unknown *FMO* gene would not be entirely unexpected, as other researchers have stated that mixed-probe, low stringency analysis of human genomic DNA indicates that one, and possibly two, more *FMO* genes remain to be characterized (344), although it might have been expected that the gene would be found on chromosome 1q along with the other five members (see Chapter 1, Section 1.6 and Chapter 3: Localization of human *FMO2* and *FMO5* genes to chromosome 1q). As a final note of caution, in this case we would be assuming that the newly discovered *FMO* gene is also primarily responsible for trimethylaminuria, whereas recent work by Dolphin *et al* (299) suggests that the *FMO* gene responsible for this disorder is *FMO3* (see Chapter 1, Section 1.11, Subsection 1.11i). If not a new *FMO* gene, another explanation might be the existence of an *FMO* pseudogene in this region. However, the rapid sequence drift of pseudogenes taken together with the Southern hybridization data theoretically makes this seem rather unlikely (this point is discussed further in Chapter 7, Section 7.12: Summary).

### 8.3 Cosmid clone 2, colony 2.2

The cosmid DNA preparation for cosmid clone 2, colony 2.2 was shown to hybridize to two regions, the heterochromatic region of chromosome 1q (1qh) and the centromere of chromosome 10 (Figure 8.2). The clone is therefore possibly chimeric and hybridizes to regions of the genome both of which are heterochromatic and thought to be devoid of transcriptionally active DNA. It is also possible (and perhaps more likely), however, that the insert of this clone is hybridizing to these two regions of the genome not because of chimerism but because these regions happen to contain sequences that share a high degree of similarity.
Figure 8.2. FISH analysis to human metaphase chromosomes with cosmid clone 2, colony 2.2 as probe. Cosmid clone 2 originates from the ICRF human chromosome 1-specific library and was selected because of its apparent ability to hybridize to an FMO probe mixture (radiolabelled FMO1, FMO2, FMO3, FMO4 and FMO5 full-length cDNAs). Colonies such as 2.2 were those shown to bind the same probe mixture upon subsequent replica plating. Southern hybridization analysis revealed that cosmid DNA from this clone hybridized very strongly to all five FMO gene sequences individually. Above we show the results of fluorescence in-situ hybridization to human metaphase chromosomes with labelled clone as probe to reveal the genomic location(s) of the insert. Regions to which the clone hybridizes appear in green on the red chromosomes. Hybridization is evident at both 1qh and the centromere of chromosome 10 (arrows on the right mark 1qh and arrows on the left mark the centromere of chromosome 10) and so the clone insert may be chimeric.
Much of the DNA sequence in these heterochromatic regions is highly repetitive in nature (the main component of so-called satellite DNA). Southern hybridization analysis showed that the insert of this clone has an exceptionally strong hybridization affinity towards all five \textit{FMO} gene sequences. It would therefore seem likely that, by chance, certain highly repetitive DNA sequences from these regions of the human genome have a high degree of homology to regions of the five \textit{FMO} genes that share most sequence similarity with each other, for example, the FAD-binding site region at the 5’ end of the genes. Such an explanation would also match with the observation that the entire insert appeared to have affinity for the \textit{FMO} probes.

\subsection*{8.4 Cosmid clone 6, colony 6.1}

This clone hybridized solely to 1p36 (Figure 8.3). It almost certainly has a high degree of sequence similarity within its insert to regions of all five \textit{FMO} sequences, based on the Southern hybridization data. 1p36 lies at the end of the chromosome and therefore includes the telomere. Along with centromeres, the telomeres make up the bulk of heterochromatic DNA in a chromosome and represent a substantial fraction of satellite DNA. Therefore, as was the case for clone 2, colony 2.2, the cosmid insert has hybridized to a region of the human genome characterized by highly repetitive DNA. Once again then, it would appear likely that these commonly repeated sequences share a coincidental homology with regions of sequence highly conserved between the \textit{FMO} genes and that this would explain how the entire clone insert hybridized to the five \textit{FMO} probes.

\subsection*{8.5 Cosmid clone 7, colony 7.1}

Hybridization was located at 1p36, the same telomeric region as for clone 6. colony 6.1 (Figure 8.4). Identical assumptions can therefore be made in explaining how the entire insert of this clone was able to strongly hybridize to all five \textit{FMO} gene sequences.
Figure 8.3. FISH analysis to human metaphase chromosomes with cosmid clone 6, colony 6.1 as probe. Cosmid clone 6 originates from the ICRF human chromosome 1-specific library and was selected on the basis of an apparent hybridization affinity to an FMO probe mixture (radiolabelled FMO1, FMO2, FMO3, FMO4 and FMO5 full-length cDNAs). Colonies shown to bind the same probe mixture upon subsequent replica plating were picked, one of which was colony 6.1. Southern hybridization analysis revealed the entire insert of this clone to be capable of strong hybridization to all five FMO gene sequences individually. Above we show fluorescence in-situ hybridization to human metaphase chromosomes with labelled 6.1 as probe to reveal the genomic origin(s) of the insert. Regions to which the clone hybridizes appear in green on the red chromosomes. Hybridization is evident at 1p36 (arrowed).
Figure 8.4. FISH analysis to human metaphase chromosomes with cosmid clone 7, colony 7.1 as probe. Cosmid clone 7.1 originates from the ICRF human chromosome 1-specific library and was selected because of its apparent ability to hybridize to an FMO probe mixture (radiolabelled FMO1, FMO2, FMO3, FMO4, and FMO5 full-length cDNAs). Colonies such as 7.1 were those shown to bind the same probe mixture upon subsequent replica plating. Southern hybridization analysis revealed that cosmid DNA from this clone hybridized very strongly to all five FMO gene sequences individually. Above we show the results of fluorescence in-situ hybridization to human metaphase chromosomes with labelled 7.1 as probe to reveal the genomic location(s) of the insert. Regions to which the clone hybridizes appear in green on the red chromosomes. Hybridization is evident at 1p36 (arrowed).
8.6 Cosmid clone 11, colony 11.1

Figure 8.5 shows that this clone hybridized to multiple sites within the genome. The insert of this clone may therefore be chimeric in nature and/or some of the regions of the genome at which hybridization took place share sequence similarity (a more likely possibility that was discussed above for cosmid clone 2). Hybridization was to the regions 1p34-35, 1q23-25, 1p36 and 18q22, the latter region indicating once more that the library may not be chromosome 1-specific. One is immediately alerted by the region 1q23-25, as this is the region shown to contain $FMO1$, $FMO2$, $FMO3$ and $FMO4$ genes. However, the telomeric location 1p36 is once again present (see 'Note' at end of Chapter 5, Section 5.18: Implications, in which it is noted that 1p36 seems consistently capable of hybridizing to $FMO$ sequences), so it seems at least as likely that repetitive sequences from this region, rather than actual $FMO$ genes from 1q23-25, are responsible for the $FMO$ probe hybridization witnessed in the Southern hybridization experiments. Indeed, given that each of the DNA fragments produced by BamH1 digestion of this clone hybridized to $FMO$ probes (Figure 7.3 and Figure 7.4 in Chapter 7), the most likely explanation for the Southern hybridization and FISH analysis data is that the genomic insert originates from a single heterochromatic region of chromosome 1 that shares sequence similarity to other heterochromatic regions of the genome (hence the multiple sites of hybridization under FISH analysis) and 1q23-25 (not entirely unexpected as if there is homology to the $FMO$ probes then one might expect hybridization to that part of the genome where the $FMO$ genes lie).

8.7 Other clones

Although the above five clones were the only ones shown in the Southern hybridization experiments to hybridize to $FMO$ probes under high stringency conditions, the other clones had shown a very weak affinity for the probes when the library was originally screened and during the replica plating experiments. Out of interest, two of these clones, clone 5, colony 5.1 and clone 10, colony 10.1, were also subjected to FISH with human metaphase chromosomes. Interestingly, although both were shown to have chimeric inserts, clone 5, colony 5.1 hybridized to 1qh and 1p36 and clone 10, colony 10.1 hybridized to 1p36. Once more then, it would seem that highly repetitive regions of the human genome were probably responsible for the isolation of these clones.
Figure 8.5. FISH analysis to human metaphase chromosomes with cosmid clone 11, colony 11.2 as probe. Cosmid clone 11 was selected from the ICRF human chromosome 1-specific library following screening with an FMO probe mixture (radiolabelled FMO1, FMO2, FMO3, FMO4 and FMO5 full-length cDNAs). Colonies such as 11.1 were those shown to bind the same probe mixture upon subsequent replica plating. Southern hybridization analysis revealed that the insert of this clone hybridized very strongly to all five FMO gene sequences individually. We show here the results of fluorescence in-situ hybridization to human metaphase chromosomes with labelled clone as probe to reveal the genomic location(s) of the insert. Regions to which 11.1 hybridizes appear in green on the red chromosomes. Hybridization was evident at three sites on chromosome 1 (chromosome 1 is the larger of the chromosomes marked by the arrows), namely lp34-35, lp36, and lq23-25, and at 18q22 (smaller of the arrowed chromosomes), and so the clone insert may be chimeric.
8.8 Summary

The primary aim of screening the ICRF human chromosome 1-specific cosmid library was to obtain regions of human genomic DNA spanning either the cluster of FMO genes (FMO1, FMO2, FMO3 and FMO4) at 1q23-24 or the FMO5 gene at 1q21. Having obtained these clones, it was intended that they may assist with determination of the FMO gene order on 1q, further understanding of how the FMO genes are regulated and perhaps identify new FMO genes in this region by low stringency hybridization analysis. Numerous clones were isolated from the library by probing with the full-length cDNAs for all five FMO genes. Unfortunately, in the case of six of the seven clones examined by FISH analysis of human metaphase chromosomes, four of which displayed exceptionally strong hybridization affinity for the FMO probes during Southern hybridization analysis, the inserts appeared to originate from heterochromatic regions (either the centromere or the telomere) of the genome known to be largely devoid of transcriptionally active DNA but rich in highly repetitive sequences. The seventh clone, which displayed a relatively weak hybridization affinity for all the FMO probes, was the exception as its insert appeared to originate solely from the euchromatic region 15q11-13. It is known that this region is deleted in individuals afflicted by the disorder Prader-Willi syndrome. A clinical case report has been published of an individual with this disorder and a deletion at 15q11-13 who was also suffering from trimethylaminuria. Trimethylaminuria is assumed to be caused by the loss of activity of an FMO enzyme, so it is intriguing to speculate whether an unknown additional member of the FMO gene family is located in this region and that cross-hybridization was taking place between a region of this gene within the clone and the FMO probes. However, while it remains a possibility that the human FMO gene family has more than five members, recent work by other researchers indicates that the likely gene candidate responsible for trimethylaminuria is FMO3. A summary of the data presented here is given in Table 8.1.
Table 8.1. Genomic origin of cosmid clone inserts and their relative hybridization affinity for *FMO* probes. The five cosmid clones listed above were originally selected from the ICRF human chromosome 1-specific cosmid library following high-stringency hybridization to a probe mixture containing full-length human *FMO1, FMO2, FMO3, FMO4* and *FMO5* cDNAs. All were shown to anneal to the same probe mixture after replica plating. A crude and subjective estimate using Southern hybridization analysis as to the relative degree to which radiolabelled individual full-length human *FMO* cDNA probes hybridized to the clones is given. This was undertaken by visual examination of the X-ray films produced by autoradiography. The number of plus (+) symbols denotes the apparent relative affinity of the probe for the clone concerned. FISH analysis to human metaphase chromosomes using the clones as probes allowed determination of the genomic origin(s) of the clone inserts. *, denotes that for clone 1, probe binding occurred on just one fragment for only one of the two colonies subjected to the Southern hybridization analysis (colony 1.1).
Chapter 9

Discussion and conclusions
In Chapter 3 (Localization of human FMO2 and FMO5 genes to chromosome 1q) I have shown that the human genes, FMO2 and FMO5, reside on the long arm of chromosome 1 (1q). At the time, these two genes were the only known members of the human FMO gene family for which a chromosome location had not yet been identified. Previously, others had used similar techniques to localize human FMO1 (104), FMO3 (171) and FMO4 (84). As, in each case, these genes were also found to reside on 1q, FMO1 to FMO5 have remained in this location since the postulated time of the duplication of their common ancestral gene, some 290 million years ago (84, 171) (See Chapter 1, Sections 1.3, Subsection 1.2: Primary structures of the mammalian FMOs, and 1.6: Genome localization of human FMO genes). Why this is so is not known, as some other gene families believed to have diverged more recently (for example, the cytochrome P450 gene family (86, 178, 180)) have members that are distributed across different chromosomes. Only further research will ascertain whether the clustering of the human FMO genes is simply a chance event, or if it is related to the molecular basis by which these genes are regulated.

We felt that research into the molecular biology of human FMOs would be assisted by the isolation of the genomic fragments on which they reside. Such fragments, we believed, would enable (among other things) a more precise localization of the FMO genes on 1q, and the determination of their relative order with respect to each other in this region. Other hitherto undiscovered FMO genes may also be discovered on such fragments, given that the entire family appears to be clustered in a relatively small region of the genome. In addition, the fragments would inevitably yield some 5’ flanking promoter sequences, for which hardly any information exists, even now. Each of the known FMO genes are expressed differently according to, for example, the tissue examined, stage of development, nutritional status and endocrine status of the organism (See Chapter 1, Sections 1.3: The family of mammalian FMOs, 1.4: FMO activity in relation to endocrine and nutritional status and 1.5: Ontogenic and tissue-specific expression of FMOs). Only by analyzing these sequences could progress be made in understanding the molecular basis of these regulatory mechanisms.

It was to this end that numerous attempts were made to screen human genomic DNA libraries for FMO genes, using the full-length cDNAs for FMO1 to FMO5 as probes. In Chapter 4 (Isolation of YACs bearing FMO-containing human genomic inserts), I described a successful attempt to isolate such fragments in the form of yeast artificial chromosome (YAC) clones. In all, fifteen YAC clones
were isolated that were shown by Southern hybridization analysis to contain one or more \textit{FMO} genes (see Table 4.7 in Chapter 4). Two of the clones were found contain \textit{FMO1, FMO2, FMO3} and \textit{FMO4} (demonstrating that these four genes, at least, are tightly linked) and four to contain just \textit{FMO5}. These clones are in the process of being further characterized by my colleagues at UCL for purposes just described. I was able to deduce from the Southern hybridization data that the gene order for \textit{FMO1, FMO2, FMO3} and \textit{FMO4} along \textit{lq} is either 1423 or 4123, in either orientation.

These YAC clones were then subjected to fluorescence \textit{in-situ} hybridization (FISH) to human metaphase chromosomes (Chapter 5: Fluorescence \textit{in-situ} hybridization analysis of YAC clones bearing human genomic sequences containing human \textit{FMO} genes). This allowed me to improve the resolution of the locations of \textit{FMO1, FMO2, FMO3} and \textit{FMO4} to \textit{lq23-24}. Furthermore, the FISH analysis revealed that \textit{FMO5} is located some distance from the other four known members of the family towards the centromere at \textit{lq21}. It is tempting to speculate whether the removal of \textit{FMO5} from the other known members of the \textit{FMO} gene family is related to the fact that \textit{FMO5} is apparently unique in being a non-drug metabolizing \textit{FMO} enzyme (140, 141).

The sizes of the inserts of three of the YAC clones were estimated by performing pulsed field gel electrophoresis (PFGE) (Chapter 6: Pulsed field gel electrophoresis of YAC clones bearing human \textit{FMO} sequences). The three YAC clones I chose to undergo this form of characterization were those I regarded as being the most suitable for further analysis: two contained just \textit{FMO5} and the other \textit{FMO1, FMO2, FMO3} and \textit{FMO4}, and each were revealed not to be chimeras from the FISH analysis. Two of the inserts were estimated to be close to 500kb, whereas the other was at least 1000kb in size. Southern hybridization of the pulsed field gel confirmed the presence of the expected \textit{FMO} genes within these three clones.

Included in my other attempts to isolate human genomic fragments containing \textit{FMO} genes was a screening of a chromosome 1-specific library constructed in cosmid vectors (Chapter 7: Screening of human chromosome 1-specific cosmid library for \textit{FMO} genes). The smaller insert size of cosmids, it was believed, would make their characterization and manipulation easier than that of YAC inserts, although there was far less chance of isolating whole \textit{FMO} genes. Ideally, it was intended that cosmid clones containing each of the individual \textit{FMO} genes would be obtained. These would then be employed as probes in FISH analysis of YACs containing multiple \textit{FMO} genes, in order to determine
the unequivocal \textit{FMO} gene order along 1q. In Chapter 7 I described how numerous cosmid clones with strong affinity for full-length \textit{FMO} cDNA probes were isolated from the library. However, subsequent Southern hybridization analysis of these clones indicated that, in all probability, their inserts did not contain \textit{FMO} genes, but rather some other genomic regions with a high degree of sequence homology to \textit{FMO} sequences. This was confirmed by performing FISH to human metaphase chromosomes with those cosmid clones shown to have the highest binding affinity for the \textit{FMO} probes (Chapter 8: Analysis of cosmid clones isolated from a chromosome 1-specific library by fluorescence \textit{in-situ} hybridization to human metaphase chromosomes). It would appear that these clones contain inserts originating from the heterochromatic regions of chromosome 1, specifically, the telomeric and centromeric regions. An exception to this was a cosmid clone that unexpectedly hybridized to 15q11-13 only, the same region that has been suggested by other workers to possibly contain an \textit{FMO} gene (264), based on the genetic analysis of an individual with Prader-Willi syndrome who also showed signs of lacking normal FMO activity in the form of trimethylaminuria (see Chapter 1, Section 1.11, Subsection 1.11i: Fish-odour syndrome/Trimethylaminuria). However, in light of the recent work of Dolphin \textit{et al} (299), showing that FMO3 is almost certainly the FMO form primarily responsible for the metabolism of trimethylamine (see Chapter 1, Section 1.11, Subsection 1.11i), I believe it highly unlikely that the insert of this particular cosmid clone contains the sequence of a new, sixth \textit{FMO} gene.

If we compare the FISH analysis results for the \textit{FMO}-containing YAC clones (Chapter 5) in which some were found to co-hybridize to regions of the genome other than 1q21 or 1q23-24, with the FISH analysis results for the cosmid clones (Chapter 8), there can be little room for doubt that the telomeric region 1p36, at least, contains sequences with a very high degree of homology to all five known \textit{FMO} genes.
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Localization of Human Flavin-Containing Monooxygenase Genes
FMO2 and FMO5 to Chromosome 1q

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The human flavin-containing monooxygenase (FMO) gene family comprises at least five distinct members (FMO1 to FMO5) that code for enzymes responsible for the oxidation of a wide variety of soft nucleophilic substrates, including drugs and environmental pollutants. Three of these genes (FMO1, FMO3, and FMO4) have previously been localized to human chromosome 1q, raising the possibility that the entire gene family is clustered in this chromosomal region. Analysis by polymerase chain reaction of DNA isolated from a panel of human–rodent somatic cell hybrids demonstrates that the two remaining identified members of the FMO gene family, FMO2 and FMO5, are also located on chromosome 1q.

The flavin-containing monooxygenases (FMOs; EC 1.14.13.8) catalyze the NADPH-dependent oxidation of a wide range of structurally diverse compounds that characteristically contain a soft nucleophilic heteroatom such as nitrogen, phosphorus, or sulphur as the site of oxidative attack (19). Substrates of the FMOs include several drugs, dietary components, and environmental pollutants, and the enzymes have been implicated in both the detoxification and the metabolic activation of a variety of xenobiotics (19). An inherited defect in the FMO-catalyzed N-oxidation of the dietary-derived amine, trimethylamine, results in the disorder trimethylaminuria, colloquially termed fish odor syndrome because affected individuals excrete relatively large amounts of the malodorous free amine in their breath, sweat, and urine (5, 12). To date, five distinct FMOs, with amino acid sequence identities between 51 and 57%, have been identified in mammals including human (11). These proteins, designated FMO1 to FMO5, each appear to be encoded by a single gene. Sequences have been published for four members of the FMO human family, FMO1 (2), FMO3 (4, 9), FMO4 (3), and FMO5 (10). It should be noted that, to conform to the recently proposed FMO nomenclature (8), the human FMO form originally termed FMO2 (3, 13) has been redesignated FMO4. However, definitions of the forms originally referred to as FMO1 (2, 13) and FMO3 (13) are unchanged. FMO1 (2), FMO3 (13), FMO4 (3) have been mapped to human chromosome 1. Regional mapping studies indicate that each of these genes is situated on the long arm of this chromosome (13), and the localization of FMO1 has been further refined to 1q23–q25 (13), suggesting that the entire FMO gene family forms part of a single gene cluster. We have recently isolated and sequenced cDNA clones that code for human orthologues of the two remaining identified members of the FMO gene family, FMO2 and FMO5 (11). We now report the chromosomal mapping, by means of the polymerase chain reaction (PCR), of the human FMO2 and FMO5 genes.

For FMO2, oligonucleotides (5’ CTCTCAGTTCATATGGCCAG 3’ and 5’ TACTGGATCTCGACAGATAAAAGGCCCAG 3’ ) were synthesized that would anneal to sequences located on either side of the translation termination codon of the gene and prime the amplification of a 250-bp DNA fragment, the majority of which was derived from sequences coding for the 3’-untranslated region of the corresponding mRNA. For FMO5, oligonucleotides (5’ CTTATTTCTGACAGATAAAAGGCC 3’ and 5’ ACA- TATTTCTGACAGATAAAAGG 3’ ) were synthesized that would prime the amplification of a 400-bp fragment of the gene consisting entirely of sequences coding for the 3’-untranslated region of the corresponding mRNA. PCRs were performed as described previously (13), and the amplification products were analyzed by agarose gel electrophoresis. Each set of primers directed the amplification from human geno-
## TABLE 1

Segregation of Human *FMO2* and *FMO5* in Relation to Chromosomal Content in Human–Rodent Hybrids

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Note. +, Human chromosome present; -, human chromosome not detected; 0, only part of chromosome present. Concordance and discordance figures do not include results from hybrids that contain only a part of human chromosome 1.
mic DNA of a DNA fragment of the expected size (Figs. 1A, lanes c and q, and 1B, lanes d and e). The oligonucleotides were also able to prime the amplification of a correctly sized fragment from the cDNA clones encoding the corresponding FMO, but not from cDNAs encoding any of the other four FMOs (data not shown), thus confirming the specificity of the amplification reactions. An analysis of a panel of human–rodent somatic cell hybrids revealed that amplified products indicative of the human FMO2 and FMO5 genes were obtained only from DNA isolated from hybrids containing human chromosome 1 (Figs. 1A, lanes f to o, and 1B, lanes f to m, respectively, and Table 1). No amplification products corresponding to human FMO sequences were obtained from DNA isolated from mouse or hamster donor cell lines (Figs. 1A and 1B, lanes d and e, respectively).

A more precise localization of the FMO2 and FMO5 genes was undertaken by analyzing three additional somatic cell hybrids that contained either the long or the short arm of human chromosome 1. Fragments derived from the human FMO2 and FMO5 genes were amplified only from DNA isolated from the hybrid (CON2) that contained the long arm of chromosome 1 and not from DNA isolated from either of the hybrids (CON5E and F4sc13cL12) containing the short arm of this chromosome (Figs. 1A, lanes r to t, and 1B, lanes p to r, and Table 1).

Our results demonstrate that both the FMO2 and the FMO5 genes are located on human chromosome 1q, the same region to which we have previously mapped the FMO1, FMO3, and FMO4 genes (13). FMO genes appear to have arisen from a common ancestral gene, via gene duplications, some 250–300 million years ago. The presence on the long arm of chromosome 1 of genes coding for all five known members of the FMO human family suggests that, subsequent to these duplication events, the entire FMO gene family has remained linked, possibly in a single clustered formation.

ACKNOWLEDGMENTS

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REFERENCES


