The human cytochrome P450 sub-family: Transcriptional regulation, inter-individual

variation and interaction networks

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Summary

The Cytochrome P450 super-family is a fundamental requirement for the viability of most life, with Cytochrome P450 proteins having been identified in organisms ranging from bacteria to man. These enzymes may be subdivided into those that metabolise purely endogenous chemicals, and those that are involved in xenobiotic metabolism. Of the latter group it can be argued that CYP3A sub-family members rank as the most important; their high expression in the liver and wide substrate specificity mean that they are clinically important in the metabolism of many therapeutic drugs, and alteration in their activity is central to many clinically-relevant drug-drug interactions.

In this review I will examine the human CYP3A enzymes, discussing their genome structure, common allelic variants and, in greatest detail, their transcriptional regulation. Through examination of these characteristics we will see both striking similarities and differences between the four human CYP3A enzymes, which may have important impacts on inter-individual response to chemical exposure. Finally, the role of nuclear receptors in regulating CYP3A gene expression, and indeed that of many other proteins involved in drug metabolism, will be examined: Such an examination will show the need to utilize a systems biology approach to understand fully how the human body responds to chemical exposure.

General Introduction

Members of the cytochrome P450 (CYP) super-family of enzymes form an essential part of the body's ability to carry out both anabolic and catabolic metabolism. Indeed, the very fact that members of this super-family have been identified in all studied organisms, from archaebacteria to humans, underlines their importance for organism survival [1]. Although all organisms possess CYP enzymes the number of CYP genes per organism is not constant, with more ancient organisms having fewer CYP genes. Indeed, it is the development of organisms with a gastro-intestinal tract that triggered a marked in CYP genes per organism, with increased chemical intake through the diet becoming a significant driver in the need to evolve new proteins capable of metabolising these chemicals [2]. Of the CYP isoforms present within an individual organism it is therefore possible to further subdivide them into those that mediate metabolism of endogenous chemicals and those that undertake xenobiotic metabolism, with members of this latter group being the focus of this review. Of the 57 CYP genes present in humans, is it really only member of families CYP1, CYP2 and CYP3 that are involved in xenobiotic metabolism. Of these, the CYP3A sub-family represents perhaps the most significant group due to two facts: First, CYP3A enzymes are the most abundant CYPs in human liver, comprising between 30-50 % of total CYP content, and hence represent the bulk of the CYP enzymes that a chemical is likely to be exposed to [3]. Second, a large active site results in substrate promiscuity, meaning that up 60 % of therapeutics in use today that are subject to metabolism are substrates for CYP3A sub-family members [4]. Taken together it can be seen that for the majority of xenobiotics CYP3A plays some role in their metabolism in humans.

Relevance of studying CYP3A gene expression

An amazing ability of the human body is its ability to handle xenobiotics, utilizing those that are beneficial to the body and removing rapidly those that might prove harmful. Indeed without such ability it is questionable whether *Homo sapiens* would have ever arisen, for the requirement of higher organisms to interact, and react, to their environment is an absolute. This metabolic capacity is not only large but flexible, with protein levels being increased in response to chemical exposure, resulting in the most efficient response to any xenobiotic. It is thus logical that to understand how the body responds to xenobiotics we must first understand how these chemicals interact with the metabolic network within the body.

An important extension of this need to be able to *understand* the molecular mechanisms of body-xenobiotic interactions is the requirement to be able to *predict* such events, and in particular those that may result in toxicity. Prediction of toxicity as a result of the bio-activation of chemicals that occurs during metabolism is relatively straightforward, and can be achieved to a good degree of accuracy using in silico programs such as DERECK, TOPCAT or HazardExpert [5-7]. However and area of increasing concern are toxic reactions caused due to interactions between co-administered chemicals. Combination chemotherapy is used increasingly in medial healthcare today, for the treatment of complex, life-threatening diseases such as HIV [8] or cancer [9], or in individuals with multiple, often long-term conditions, such as the elderly [10, 11] or during long-term prophylaxis of psychiatric disorders [12, 13]. Co-administration of multiple drugs that are metabolised by the same enzyme system(s) to a single individual may potentially result in altered pharmacokinetics for some, none, or all of the co-administered compounds. If these effects occur to such an extent that clinical efficacy is lost, or toxicity produced, then such interactions are usually rapidly reported in the literature. Indeed, it has been estimated that adverse drug reactions are

the fourth largest killer in the Western World, and as such are a major health concern. Such drug-drug interactions may occur through two major mechanistic routes, inhibition of enzyme action or activation of gene transcription. Inhibition is a widely accepted problem, and much research has been undertaken in this area; the interested reader is directed to these recent reviews [14-16]. The focus of this review will be on the increase in protein number caused by activation of gene transcription. It should be noted that increased protein expression can also occur by chemical-mediated increases in transcript (e.g. clotrimazole [17] or protein (e.g. triacetyloleandomycin [18]) stability, but these are more the exception rather than the rule and will not be discussed herein.

The presence of a wide-substrate binding profile for an enzyme is a direct correlate with predilection for drug-drug interactions and hence it is not surprising that CYP3A enzymes are subject to large number of such interactions. Table 1 presents a selection of established drug-drug interactions involving activation of CYP3A gene transcription. An interesting point to note is that several of these are not 'drug'-'drug' interactions in the true sense as the inducing agent is not a therapeutic agent; this underlines the importance in understanding the molecular mechanisms of drug-drug interactions as individuals may be exposed to many of the combinations without their physicians, or indeed their own, knowledge.

[TABLE 1 ABOUT HERE]

CYP3A family members

Despite its high biological significance with regards to xenobiotic metabolism, the CYP3A sub-family is relatively compact, comprising only four members in humans: CYP3A4, CYP3A5, CYP3A7 and CYP3A43 [19]. Following the basic definition of

sub-family membership, the sequences for these proteins demonstrate at least 70 % identity, with the exact matches being given in Table 2.

[TABLE 2 ABOUT HERE]

A particular feature of CYP-biology in general, and one that is particularly relevant to the CYP3A sub-family in particular is that large inter-individual variations in enzyme expression have been reported [20-23]. Such variation is probably due to a combination of chemical-mediated induction/inhibition of CYP activity (e.g. environment) and the presence of activity/expression polymorphisms (e.g. genetic). The exact input of these two factors is a matter of debate, with estimates for the genetic contribution towards the variation in CYP3A expression ranging from 60 % to 90 % [24]. A large compendium of CYP alleles is held by the official allele nomenclature committee (http://www.imm.ki.se/CYPalleles/), which currently lists 40 variants for *CYP3A4*, 24 for *CYP3A5*, 7 for *CY3A7* and 5 for *CYP3A43*. However a survey of the literature reveals the majority of these variants are either of little biological significance or low frequency, and hence would not act as a major contributor to the observed inter-individual variability in CYP3A activity [22, 25-29]: Selected variants with the largest potential biological impact are discussed in detail within the relevant sections below. This lack of 'major' allelic variants to account for inter-individual variation, as for example seen in CYP2D6 [26], suggests that inter-individual variation in CYP3A activity is the result of one of three scenarios. First that the large number of 'minor' variants combine together to produce the total variation in CYP3A activity that is observed in the population. Second that environmental impact is the major player in determining CYP3A activity, and individual exposure to CYP3A inhibitors/inducers

determines CYP3A activity. Third, that genetic variation exists outside the CYP3A genes, which impacts upon their expression. Of these possibilities, the first seems unlikely as the frequency of these variants in combination still appears too low to explain all the observed variation in activity, as will be discussed below. However, the second and third possibilities are equally valid, and I suspect that the true answer is a combination of both; the exact ratio of these inputs being as yet undetermined.

Whereas inter individual variation in CYP3A activity is generally accepted, investigations for potential sex differences in expression are much more equivocal. In two separate studies erythromycin *N*-demethlyation has been reported to be greater in females, by 24 % [30] and 36 % [31], suggesting that a slight sex difference may exist. However, the difficulty in removing confounding factors from such studies means that such data must be treated with caution.

When studying the CYP3A sub-family it is perhaps fair to say that equal importance should not be given to all family members. Although all individuals tested to date appear to express CYP3A activity [19], the different isoforms input differently into this activity: Each isoform will be discussed below in more detail, along with the major polymorphisms associated with the corresponding gene.

CYP3A4

CYP3A4 transcripts are undetectable in foetal liver [32], but rapidly rise after birth whereupon CYP3A4 becomes the predominant liver CYP in the majority of adult livers [4, 33]: Due to this fact CYP3A4 is the best studied of the CYP3A sub-family members in humans.

The wide substrate binding profile and catalytic activity of CYP3A4 results in a number of possible biotransformation reactions, encompassing C- and N-oxidations, N- and O-dealkylations, nitro reductions and dehydration reactions [19]. Recent

crystallographic study of CYP3A4 [34], combined with homology modelling [35-37] and mutagenesis of recombinant CYP3A4 [38-43] has identified key residues within the active site that are responsible for reaction catalysis.

A number of CYP3A4 allelic variants have so far been identified that may contribute to the known inter-individual expression of CYP3A [27, 28, 44-47]. Of these, some variants have been shown to result in the total ablation of holoenzyme production (e.g. R130Q and P146L [45]), whereas others impact directly on enzyme activity (e.g. L373F [45]and F189S [46]). However, the relatively low frequency of these alleles means that the overall input of individual variants towards the observed variation in CYP3A activity is questionable [48]. For example, one of the more widely expressed variants is F189S, yet this variant only has an allelic frequency of 2 % in Caucasians [46]. Recently, Eiselt and colleagues identified eight CYP3A4 variants, of which only four have a substantial effect of CYP3A4 activity. The combined heterozygote frequency of these eight variants was only 7.5 % in the European population [45], underlying the disparity between the impact of the identified CYP3A4 variants on CYP3A activity and the much larger total variation in CYP3A activity observed in the general population.

CYP3A5

In parallel with CYP3A4, significant expression of CYP3A5 is not associated with the developing foetus, with low level expression being detected in less than 10 % of human foetal livers [49]. However, in contrast to CYP3A4, which is expressed in all adult human livers, CYP3A5 expression appears to be limited to approximately one-quarter of individuals [49]: In these individuals, however, the level of CYP3A5 is often equal to, or exceeds, that of CYP3A4, making it a significant player in determining overall CYP3A activity for these individuals [50]. Given the known differences in

metabolic capacity between CYP3A isoforms [51-55], such variation in expression may have a potentially large impact on the wide inter-individual variation in CYP3A activity observed in the liver [4]. It is also worthy of note that CYP3A5 is expressed in several organs in which CYP3A4 is not highly expressed, for example skeletal muscle [56], and hence differences in metabolic profile between liver and these organs may exist.

The very fact that CYP3A5 is expressed in only approximately one-quarter of adult livers suggests the presence of allelic variants for this gene. As stated earlier, the majority of identified SNPs for CYP3A5 are of little population significance, due either to their low frequency or small biological impact [29]. However, Kuehl and colleagues identified a family of allelic variants for which both a significant population and biological effect exists. They identified three splice acceptor site polymorphisms that resulted in the potential inclusion of mini-exons between exons 3-4, 4-5 and 5-6 (designated exons 3B, 4B and 5B) [57]. Inclusion of these mini-exons introduces a stop codon in the CYP3A5 transcript, thus producing a truncated, non-functional CYP3A5 protein [57]. A solid hypothesis would therefore be that the variants including these mini-exons (CYP3A5*3 SV1, 2 and 3) are actually held by the majority of individuals and result in low/negligible CYP3A5 expression, with the minority expressing the wild type, fully functional, variant. Subsequent screening of diverse ethnic groups has suggested that this allelic variant (CYP3A5*3) may represent a good marker for overall CYP3A activity, and although it does not account for all non-expression of CYP3A5 may be of some use in prospective screening [58-60].

CYP3A7

Whereas CYP3A4/5 are the predominant CYP3A isoforms in adult liver, in the developing foetus CYP3A7 is the predominant CYP3A expressed, and in an inverse relationship to CYP3A4/5, levels of CYP3A7 decrease rapidly at birth [32, 33, 61, 62].

Approximately one-half of adults have detectable levels of CYP3A7 within the liver, although in general this is at lower levels to both CYP3A4 and CYP3A5 and probably has a negligible input into CYP3A-mediated metabolism [32]. However, approximately 10 % of adults do have significant levels of CYP3A7; this increased expression being associated with the CYP3A7*1c allele, which affects the PXR response element in the CYP3A7 promoter. In these individuals CYP3A7 may contribute up to one third of the CYP3A content of the adult liver [63], and thus have a significant input into CYP3A-mediated metabolism. Liver is not the only organ that shows significant adult expression of CYP3A7, with an increase in endometrium and placental expression of CYP3A7 being observed in pregnant women [64] and during the secretory phase of the menstrual cycle [32]. It has been proposed that the increased CYP3A7 expression during pregnancy may be foeto-protective, reducing 6β- and 16α-hydroxylation of steroid precursors, which if unchecked could result in dysregulation of steroid levels in the developing foetus [65].

CYP3A43

In 2001 a new member of the human CYP3A sub-family was cloned, termed CYP3A43 [66-68]. CYP3A43 appears to be expressed in a similar range of tissues to other members of the CYP3A subfamily (e.g. liver, kidney, pancreas, testes and prostate) and has been identified in both adult and foetal samples. However, the expression of this isoform appears to be at very low levels in comparison to CYP3A4/5/7, with adult hepatic expression being only approximately 0.1 % and 2 % of mean CYP3A4 and CYP3A5 transcript levels respectively [67]. In addition, studies on the metabolic capability of CYP3A43 demonstrate very low 6β-hydroxylation activity towards testosterone, suggesting a weakly metabolically active isoform. This poor metabolic activity has been attributed to six amino acid substitutions in the active site of

the enzyme compared to CYP3A4 [66]. The total identity of CYP3A43 to other members of the CYP3A sub-family is low, on average 73 % (Table 1), just qualifying it as a member of this sub-family. This combination of low identity, low level expression and low activity means that he biological significance of CYP3A43 with regards to total human CYP3A activity is probably negligible.

CYP3A gene structure

Examination of the genomes of divergent organisms such as plants and fungi has revealed that many of the CYP genes are present in clusters on chromosomes [69, 70]. Such clustering presents strong evidence that they originated via gene duplication events [70], either as discrete events or as part of whole genome duplications [71, 72]. The CYP3A sub-family of genes is no exception of this rule, and exist as a cluster on human chromosome 7q21-q22.1 [73, 74]. The *CYP3A4*, *CYP3A7* and *CYP3A5* genes lie in a head-to-tail configuration in the order listed, with *CYP3A43* located in the reverse orientation, 44.8 Kb away from *CYP3A4*. Two non-processed pseudogenes exist (*CYP3A5P1* and *CYP3A5P2*) in the regions between CYP3A5 – CYP3A7 and CYP3A4-CYP3A7 respectively, also in head-to-tail orientation [73]. Finta and Zaphiropoulos reported a CYP3A7 derived transcript that contained exons from the CYP3A5P1 pseudogene, suggesting evolution within the CYP3A gene cluster may occur through the capture of downstream exons in addition to complete gene duplication events [73].

In general, the basic structure of the CYP3A genes is the same for each member, comprising of 13 exons with a processed transcript size of approximately 2 Kb (Table 3).

[TABLE 3 ABOUT HERE]

As can be seen from table 3, each human CYP3A gene has relatively short 5'
untranslated regions, with a mean 5' UTR length of 101nt, considerably below the
average 5' UTR length for human transcripts of 150nt [75]. Interestingly, the 3' UTR
lengths for human CYP3A genes is quite variable, ranging from 111nt in CYP3A5 to
1152nt in CYP3A4, compared to the human mean 3' UTR length of approximately
500nt [75]. The molecular rationale behind these differences in 3' UTR length is
currently unknown, but as UTRs commonly contain regulatory regions that effect
transcript stability or translation efficiency it is possible that these differences are
important in the functionality of the CYP3A transcripts and their ability to produce
CYP3A protein. In silico analysis of the 3' UTR regions of the human CYP3A genes
using the miRanda algorithm (http://microrna.sanger.ac.uk/index.shtml) reveals a
number of putative micro-RNA binding sites that may underlie the molecular
functionality of CYP3A 3' UTRs [76, 77]. It should be noted that such prediction does
not denote function, with no empirical evidence for any of these sites having yet been
demonstrated. However, the conservation of sites across evolutionary time is a good
predictor of biological significance, and several of these sites are conserved between
humans and chimpanzee's (5.5M years divergence [72]), with some even conserved
between humans and mice (40M years divergence [72]); the total number of predicted
microRNA sites, with details on those conserved between species are presented in table
4. The area of microRNA regulation of gene expression is a rapidly expanding area, and
as shown above has exciting potential implications for the expression of CYP genes.

[TABLE 4 ABOUT HERE]

Common regulatory elements for CYP3A sub-family members

A large number of studies have now been undertaken to examine the molecular mechanisms underlying xenobiotic-mediated activation of CYP3A gene expression. Whereas the majority of this work has focussed on *CYP3A4* as it encodes the major CYP3A protein in the majority of adult human livers, sufficient work has been undertaken on the regulatory elements of CYP3A5 and CYP3A7 that a comparison is worthwhile; indeed, by studying common elements we may learn more about the general mechanism through which the body responds to chemical exposure. Little or no work has been undertaken on the CYP3A43 regulatory regions and, as stated previously, the biological significance of this isoform is limited: CYP3A43 will therefore not be examined further in this section.

CYP3A regulatory region overview and general transcription factors binding

The proximal promoter regions of *CYP3A4*, *CYP3A5* and *CYP3A7* share a high degree of identity. Over the first 1Kb upstream from the transcription start site CYP3A4 and CYP3A7 exhibit a remarkable degree of identity, 91 % [78]. Indeed, this region of identity extends beyond the proximal promoter to the upstream xenobiotic-response enhancer module (XREM; -7836 bp to -7607 bp), with identify over this region being 90 % [79, 80]: After this point the sequences diverge sharply, with identity dropping to approximately 25 %. Such a high degree of similarity is highly suggestive that modes of regulation for these two genes are shared, as indeed there is much evidence to suggest this is the case [79, 81].

By comparison the first 1.5 Kb of the CYP3A5 proximal promoter shares only 60 % and 59 % identity with the corresponding CYP3A4 and CYP3A7 regulatory regions [68, 82]. The region first identified by Jounaidi and colleagues as the CYP3A5 proximal

promoter is in fact the promoter of the unprocessed pseudogene CYP3A5P1 [68, 83];

however as these two regions share over 90 % identity the conclusions drawn by

Jounaidi and colleagues are still valid for the correctly identified CYP3A5 proximal

promoter. Closer examination of the CYP3A5 proximal promoter identifies a breakpoint

at approximately 700 bp upstream of the transcription start site past which identity with

CYP3A4 and CYP3A7 promoter sequences drops rapidly [68]. This is suggestive that a

gene modification event has occurred at some point in evolution, such as a large

deletion/insertion or gene conversion with a non-CYP3A gene, resulting in this

dramatic cut-off in sequence identity.

A second difference between CYP3A5 and CYP3A4/7 is that whereas both

CYP3A4 and CYP3A7 proximal promoters contain a classical TATAA-box and basic

transcription element (BTE) [78, 80], the CYP3A5 proximal promoter has a modified

TATAA-box (CATAA) and BTE [83], which is often associated with lower

selectivity/multiple positions for the transcription start site. Work by Iwano

demonstrated that both Sp1 and Sp3 were able to bind to the BTE (-66 bp to -45 bp) of

CYP3A5, confirming their unpublished work suggest that Sp family members could

bind to the BTE of CYP3A4 and CYP3A7 [83].

Auxiliary transcription factors

Examination of the proximal promoters for *CYP3A4*, *CYP3A5* and *CYP3A7* reveals

a number of conserved transcription factor binding sites. Before describing these sites in

detail it should be noted that the literature in this area is somewhat confusing and should

be taken with a degree of caution due to the mixture of techniques used to identify these

sites. Many papers quote in silico identification of binding sites, with no empirical

evidence for functionality, whereas others have used a host of molecular techniques to

show functionality: The reader is thus warned to treat each assignment with caution

until the experimental data is presented to confirm a putative binding site assignment. Figure 1 shows the suggested transcription factor binding sites for the CYP3A4/5 and 7 regulatory regions, discriminating between those for which only an in silico annotation exists, and those for which empirical binding data has been shown.

Initial in silico examination of the CYP3A4 proximal promoter revealed putative transcription factor binding sites for Octamer binding protein (Oct1), CCAAT-binding protein (CP1), activator protein 3 (AP3), and the liver-enriched transcription factors HNF-4 and 5 [78]. Deletion construct analysis of the CYP3A4 proximal promoter (-1240 bp to +11 bp) by Ourlin and colleagues demonstrated the effect of C/EBPα and DBP on the CYP3A4 proximal promoter, although exact interaction sites were not identified [84]. Subsequent DNase I footprinting analysis of the initial 250 bp of the CYP3A4 proximal promoter has confirmed protein:DNA interactions within several of in silico predicted regions, as well as adding putative binding sites for sites for specificity protein 1 (Sp1) and activator protein 2 (AP2) [85]. Site-directed mutagenesis has been used to confirm functionality for a few sites within the CYP3A4 proximal promoter; Sp1 (-104 bp to -97 bp [85]), C/EBPα (-132 bp to -121 bp [85, 86]) and HNF3 (-195 bp to -186 bp [85]). Ablation of these sites resulted in either decreased basal expression (C/EBPα and HNF3), or reductions in drug-mediated activation of CYP3A4 expression (C/EBPα, HNF3 and SP1). The involvement of another liver-enriched transcription factor (HNF4α) in regulation of CYP3A4 gene expression was demonstrated by Tirona and colleagues, who used conditional transgenic mice to show that this factor was import for both basal and drug-mediated induction of CYP3A4 [87]. Further examination of the regulatory regions of CYP3A4 revealed that this control was through direct binding to HNF4 binding elements located within the XREM located -7785 bp to -7772 bp relative to the transcription start site [87]. In 2004, Matsumura and

colleagues identified a second enhancer module for CYP3A4, the constitutive liver enhancer module (CLEM4) [88]. The location of this unit (-11.4 Kb to -10.9 Kb) is within a region of poor sequence conservation between members of the CYP3A gene family, and hence the CLEM4 unit appears to be specific for CYP3A4. Supershift analysis over this region identified binding sites for HNF1α, HNF4α, USF1 and AP1, all of which were required for maximal enhancer activity. Such a finding is at odds to the work of Tirona who demonstrated that in transgenic mice HNF1 played no role in CYP3A gene expression [87]; at present it is not clear if this difference is due to species-specific regulation or compensation for loss of HNF1 by other factors in the knock-out animals.

Due to the high sequence identity between the proximal promoters of CYP3A4 and CYP3A7 it is logical to presume shared mechanisms of regulation, and therefore transcription factor binding sites. Several studies have produced evidence to support such an hypothesis [79, 81, 89, 90], although it should be noted that such data is not unequivocal, with some differences in regulation between CYP3A4 and CYP3A7 being noted [91]. Little experimental work has been done to characterise the molecular mechanisms underlying CYP3A7 regulation, and hence although conserved transcription factor binding sites can be observed between CYP3A4 and CYP3A7 proximal promoter (see Figure 1) such comparisons must be treated with caution. Work by Ourlin and colleagues has confirmed the action of C/EBPα on the CYP3A7 proximal promoter, mirroring the interaction between this factor and the CYP3A4 proximal promoter, but the exact interaction site was not determined [84].

As noted previously, CYP3A7 is predominantly expressed in the developing foetus, whereas CYP3A4 is adult-specific; considering the high identity between the regulatory regions of these genes a valid question is what underlies this difference in temporal

expression? The previously described CLEM4 enhancer unit of CYP3A4 does not exist in the corresponding genomic location upstream of the CYP3A5/7/43 genes. As the CLEM4 unit appears to have implications for basal expression, as opposed to drug-activated expression, then it is possible that this unit may play a role in CYP3A4-specific expression in humans [88].In addition, work by Saito and colleagues identified an NF-KB-like element within the CYP3A7 promoter (-2326 bp to -2297 bp) that is ablated by a single base-pair change in the CYP3A4 promoter [92]. This NF-KB-like element was shown to bind Sp1 and Sp3, two factors that are often associated with the basal regulation of gene expression [93]. The activity of this NF-KB-like element was also shown to be modulated indirectly through the action of HNF3 and upstream stimulatory factor 1 [92]. In addition,

In silico analysis of the CYP3A5 gene regulatory regions has revealed a number of putative binding sites in common with CYP3A4 and CYP3A7 (Figure 1). In addition, Iwano and colleagues have demonstrated that the predicted inverted CCAAT-box (-75 bp to -71 bp) was functional [83]. CCAAT-boxes are capable of interacting with a number of auxiliary transcription factors, including NF-Y, NF-1 and C/EBP family members and are important in basal gene expression. Iwano and colleagues demonstrated that NF-Y had the highest affinity for the CYP3A5 CCAAT-box, and that this element showed co-operative binding with the BTE-associated Sp family members

Ligand-activated transcription factors

Ligand-activated transcription factors are central to the ability of genes encoding proteins involved in ADME to respond to chemical exposure. These transcription factors act as xenosensors and, once activated by the presence of ligand, cause an increase in the expression of a target gene set responsible for the efficient processing of the stimulating xenobiotic [94]. The largest family of ligand-activated transcription

factors implicated in regulating drug metabolism are the nuclear receptors, a super-family of 48 proteins in man [95]. Amongst these proteins are several liver enriched transcription factors such as the HNFs, whose role in regulating CYP3A gene expression has been covered in the previous section. However, in terms of *controlling* and *co-ordinating* the response to xenobiotics then the most important factor, with respect to CYP3A genes, is the pregnane-X receptor (PXR; NR1I2). PXR was first identified in mouse by Kliewer and colleagues [96], through scanning of a mouse liver cDNA library for sequences similar to the ligand-binding domain of known nuclear receptors. Ablation of PXR expression in transgenic mice results in reduced basal expression of CYP3A11, the murine orthologue of CYP3A4, and the complete loss of pregnenalone-16α-carbonitrile (PCN)-mediated activation of CYP3A11 expression [97].

PXR has now been identified in several other species including representatives from the rodentia (mouse [96] and rat [98]), lagormopha (rabbit [99]), carnivora (dog, predicted), bovidae (cow, predicted) and primates (macaque [100] and chimpanzee, predicted). Simultaneous identification of human PXR by three groups has led to some confused nomenclature, with PXR, PAR and SXR being used interchangeably [101-103]: PXR will be used throughout herein for clarity. Examination of the chicken genome revealed a nuclear receptor (chicken xenobiotic receptor, CXR) with sequence identity similar to both PXR (mean 40 % identity with rat, mouse and human PXR) and another, closely related nuclear receptor the constitutive androstane receptor (CAR; NR1I3) (mean 40 % identity with rat, mouse and human CAR) [104]. As no orthologue for PXR or CAR exists in chicken it has hence been postulated that CXR represents an evolutionary antecedent [105, 106], thus putting the divergence of the PXR and CAR genes at after the divergence between aves and mammalian some 310 million years ago

[72]. A deposited sequence for zebrafish PXR exists within GenBank, which would argue against such a timescale of divergence. However, analysis of this sequence suggests that while it is more closely related to PXR (mean 40 % identity with rat, mouse and human PXR) than CAR (mean 30 % identity with rat, mouse and human CAR), resulting in its designation as zebrafish PXR, it is shares 40 % identity with CXR. The latter observation would therefore be consistent with CXR, and indeed zebrafish PXR, representing antecedents of the PXR/CAR xenosensing receptors in mammals.

PXR binds to DNA as a heterodimer with RXRα [107], and may utilize response elements consisting of two half sites of AGGTCA, arranged as either an everted repeat separated by six nucleotides (ER6), or a direct repeat separated by three (DR3) or four (DR4) nucleotides. The exact choice/binding efficiency at these elements appear to be dependant upon both the species [101, 108, 109] and the genomic context of the element [110]. Examination of the regulatory sequences of CYP3A4 reveals an ER6-type PXR binding site (PXRE) present at -169 bp to 152 bp [101-103], and ablation of this site reduces xenobiotic-mediated activation of CYP3A4 gene expression [111]. In addition to this PXRE within the proximal promoter of CYP3A4, work by Goodwin and colleagues identified two distal ER6-type PXREs (-7738 bp to -7717 bp) and (-7698 bp to -7682 bp) within the XREM [112]: Indeed, in vivo reporter gene assays suggest that this element is of more import in determining CYP3A4 gene expression, and the activation of its transcription by xenobiotics [113].

Given the sequence conservation between CYP3A4 and CYP3A7 regulatory regions it is perhaps not surprising that a functional ER6-type PXRE is present in the proximal promoter of CYP3A7 [81], and this gene also utilizes an XREM containing ER6-type PXREs [79]. Regulation of CYP3A5 gene expression by PXR has been more equivocal

with initial reports showing little activation of CYP3A5 expression by prototypical PXR ligands [114, 115]. However, a recent report has confirmed the functionality of the ER6-type PXRE within the proximal promoter of CYP3A5 [116] that was first suggested by Iwano and colleagues [83]. Thus, the regulation of drug-mediated activation of CYP3A gene transcription by PXR has now been demonstrated to be conserved through the three major human CYP3As. Crucially, the distal XREM appears to be absent from the CYP3A5 gene; as this is necessary for maximal activation of CYP3A4 and CYP3A7 gene expression in response to xenobiotics this may explain why PXR-mediated activation of CYP3A5 gene expression has been controversial [68].

In addition to PXR binding to the promoters/enhancers of human CYP3A genes it should be noted that promiscuity in response element specificity exists, with several other nuclear receptors capable of binding to ER6, DR3 and DR4 elements. Of particular note is the previously mentioned CAR. The interested reader is referred to the following reviews that extensively cover the interplay between PXR and CAR and their co-ordinate regulation of CYP3A genes [117-121]. The implications of this promiscuity are twofold. First, that biological redundancy exists within the xenosensing systems within the body, acting as a 'metabolic safety net' to ensure the most efficient response to any chemical stimulus [122]. Second, this potential cross-talk results in an interaction network, whereby the relative expression of receptors and/or affinity of ligand/response element determines the gene set activated by a specific chemical [123]. This interaction network would thus be capable of fine tuning the response to any particular stimulus far above what could be achieved with a series on non-interacting nuclear receptors. Indeed recent evidence suggests that nuclear receptors regulate the expression of each other, further increasing the complexity of such interaction networks [124-127]. The sum total of this interaction network may well go some way to explain the observed inter-

individual variation in CYP3A activity, with a host of minor changes (both environmental and genetic) to different factors within the network combining to produce the variable expression in CYP3A genes.

Conclusion

The genetic regulation of the cytochrome P450 genes is nearing completion with respect to characterisation. The completion of many genome sequencing projects, particularly that of four higher mammals, along with the number of at, or nearing draft status means that the *coding* of the regulatory regions for mammalian CYP genes is now known. In silico analysis has allowed the *prediction* of control mechanisms from this known coding sequence, and finally molecular analysis have allowed the *functionality* of these predicted mechanisms to be examined. Having characterised the functional interactions occurring within the CYP regulatory regions we are now left with perhaps the greatest challenge, understanding how all these factors *interact* together to produce the ultimate expression of CYPs (and indeed other proteins involved in drug metabolism), and the regulation of this expression in response to stimulus. The interactions of nuclear receptors are a good example of such an interaction network, and it is perhaps through the continued study of these ligand-activated transcription factors that we will begin to understand more about how the interactome actually works. Thus we are entering an exciting period of discovery in drug metabolism, where work will evolve from the study of the individual to the study of the system. It is this 'systems biology' that will yield the ultimate benefits from the rigorous characterisation work currently being completed; the ability to *model* and *predict* human biological responses to chemical stimulus.

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Table 1: Examples of CYP3A-mediated drug-drug interactions elicited through induction

Inducing Chemical	Victim Drug	Clincial Effect	Reference
Phenytoin	Felbamate	⇑ Clearance	[128]
St Johns Wort	Indinavir	⇓AUC, ⇓Cmax	[129]
St Johns Wort	Cyclosporin	⇓ Plasma levels	[129]
Rifampicin	Cyclosporin	⇓ Plasma levels	[130]
Rifampicin	Quinidine	⇑ Clearance	[131]
Rifampicin	Efavirenz	⇓ AUC	[132]
Troglitazone	Terfenadine	⇓ Plasma levels	[133]
Troglitazone	Paracetamol	⇑ Formation of NAPQI	[134]

Table 2: Amino acid identity in the CYP3A sub-family

	CYP3A5	CYP3A7	CYP3A43
CYP3A4	84%	88%	75%
CYP3A5		81%	75%
CYP3A7			70%

Amino acid sequences for CYP3A4 (NP_059488, 503aa), CYP3A5 (NP_000768, 502aa), CYP3A7 (NP_000756, 503aa) and CYP3A43 (NP_073731, 504aa) were aligned using DiAlign (http://www.genomatix.de/) and sequence identity calculated.

Table 3: Human CYP3A Gene Structure

	CYP3A4	*CYP3A5*	*CYP3A7*	*CYP3A43*
Number of Exons	13	13	13	13
Ref transcript and length	NM_017460 2768nt	NM_000777 1707nt	NM_000765 2080nt	NM_022820 2167nt
5' non-coding exon?	No	No	No	No
5' UTR length	105nt	88nt	106nt	104nt
3' UTR length	1152nt	111nt	463nt	549t

UTR = untranslated region

Table 4: Predicted microRNA binding sites in human CYP3A 3' UTRs

Gene	Transcript	3'UTR length	Predicted miRNA sites	Number of miRNAs that can bind to these sites	Conserved sites	Conserved miRNA	Position of conserved site	Conserved species
CYP3A4	ENST00000336411	1152nt	7	7	3	hsamiR330	3-25	Hs + Pt
						hsamiR152	128-151	Hs + Pt
						hsamiR361	281-303	Hs, Pt + Mm
						dremiR27e	593-614	Hs + Pt
CYP3A5	ENST00000222982	111nt	2	4	2	hsamiR520g	2-25	Hs + Pt
						dremiR216b	41-62	Hs + Pt
CYP3A7	ENST00000336374	463nt	7	10	1	miR27b		Hs, Pt + Mm
CYP3A43	ENST00000222382	549nt	4	6	2	hsamiR488	53-75	Hs + Pt
						mmumiR350	149-168	Hs + Pt

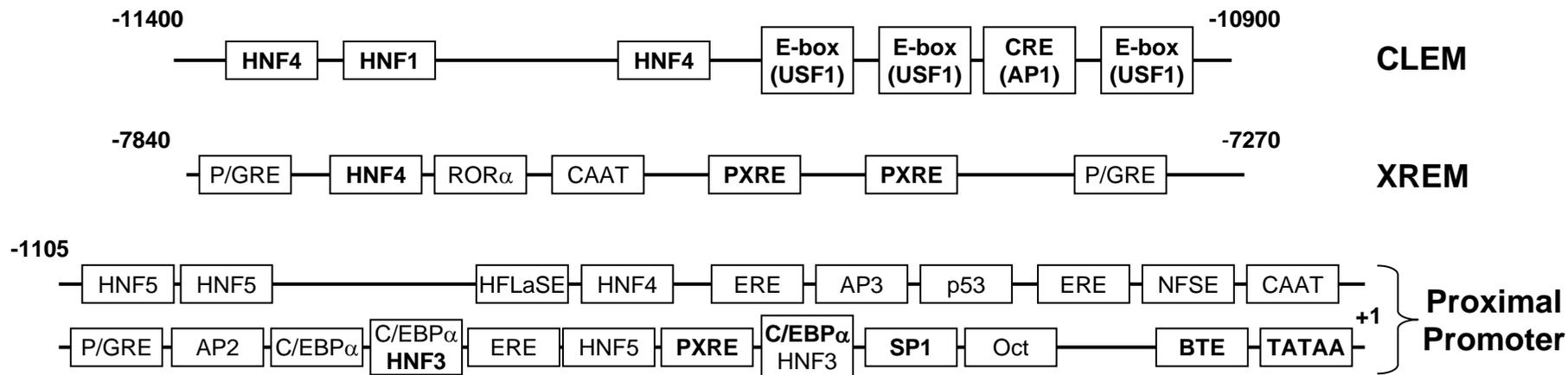Data derived from http://microrna.sanger.ac.uk/index.shtml and http://www.microrna.org/ and shows the total number of predicted miRNA binding sites per UTR, the microRNAs that can bind to these sites, plus details of micoRNA binding sties conserved across species . Hs = *Homo sapiens*, Pt = *Pan troglodytes* and Mm = *Mus musculus*

Figure Legends

Figure 1: Known and putative transcription factor binding sites within the regulatory regions of human CYP3A genes. The major identified regulatory regions for CYP3A4/5 and 7 are presented, along with putative transcription factor binding sites. Where such sites have been shown to be functional through molecular analysis the site is labelled in bold to denote this. For sites where multiple transcription factors may bind factors that have been shown to interact with this site are included in parentheses. Data from [78-80, 82-85, 87, 88, 90, 92, 112, 135]

CYP3A4

-11400   -10900

| HNF4 | HNF1 | | HNF4 | E-box (USF1) | E-box (USF1) | CRE (AP1) | E-box (USF1) | **CLEM**

-7840   -7270

| P/GRE | HNF4 | RORα | CAAT | PXRE | PXRE | P/GRE | **XREM**

-1105

| HNF5 | HNF5 | | HFLaSE | HNF4 | ERE | AP3 | p53 | ERE | NFSE | CAAT |

| P/GRE | AP2 | C/EBPα | C/EBPα HNF3 | ERE | HNF5 | PXRE | C/EBPα HNF3 | SP1 | Oct | BTE | TATAA | +1

Proximal Promoter

CYP3A5

-1105

| ½ GRE | Oct | GRE | ½ GRE | HFLaSE | HNF4 | ERE | AP3 | p53 | ERE | NFSE | CAAT |

| P/GRE | C/EBPα | C/EBPα HNF3 | ERE | HNF5 | PXRE | Oct | CAAT (NF-Y) | BTE | CATAA | +1

Proximal Promoter

CYP3A7

-7840   -7270

| P/GRE | HNF4 | RORα | CAAT | PXRE | PXRE | P/GRE | **XREM**

-2326   -2297

| NFKB-like (Sp1/Sp3) | **NFKB-like element**

-1105

| HNF5 | HNF5 | | HFLaSE | HNF4 | ERE | AP3 | p53 | CAAT |

| P/GRE | C/EBPα | C/EBPα HNF3 | ERE | HNF5 | PXRE | C/EBPα HNF3 | Oct | CAAT | BTE | TATAA | +1

Proximal Promoter