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Lessons from the *CYP3A4* Promoter

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There is considerable interest in determining the molecular basis for human variation in drug response. Investigations over the past 20 years have largely focused on identifying polymorphisms in genes that encode drug metabolism enzymes. Significant progress has been made for many of the cytochromes P450, including CYP2D6, CYP2C19, and CYP2C9 (http://www.imm.ki.se/CYPalleles/). However, the molecular basis for individual variation in CYP3A4 has remained elusive. This is unfortunate, because CYP3A4 is the most abundant hepatic and intestinal cytochrome P450, catalyzes the metabolism of more than half of all drugs, and represents the primary route of elimination for many drugs. Some studies have reported a 10-fold variation in clearance of CYP3A probe drugs (Floyd et al., 2003; Rogers et al., 2003), although 90-fold variability in CYP3A4 protein expression has been reported in liver (Lamba et al., 2002a). Some of the variation occurs because in the general population, CYP3A activity can be influenced by concurrent administration of CYP3A inhibitors and inducers.

Some variation in CYP3A activity stems from the fact that there are four human *CYP3A* genes (*CYP3A4, CYP3A5, CYP3A7*, and *CYP3A43*) in the 231-kb *CYP3A* locus located on chromosome 7q21–22.1 (Finta and Zaphiropoulos, 2000). The molecular basis for polymorphic expression of CYP3A5 and CYP3A7 in adults has been described (Kuehl et al., 2001), and CYP3A43 is expressed at too low a level to contribute significantly to hepatic CYP3A activity (Koch et al., 2002). Thus, the continuing quest is to determine the genetic basis for variable expression of CYP3A4, the isoform believed by many to be the major source of CYP3A-mediated drug metabolism.

Ozdemir et al. (2000) reported a significant genetic component to variable CYP3A expression. Several groups resequenced the *CYP3A4* coding region in attempts to identify single nucleotide polymorphisms (SNPs) or insertions/deletions (Sata et al., 2000; Eiselt et al., 2001; Lamba et al., 2002b). However, none of the SNPs are polymorphic, with frequencies of less than 1% in the populations studied (http://www.imm.ki.se/CYPalleles/), and thus cannot be the major factors influencing CYP3A expression. Resequencing of the *CYP3A4* promoter has identified multiple variants (Kuehl et al., 2001; Lamba et al., 2002b), including one common variant allele, a −392A→G transition that has a higher allelic frequency in African Americans than in white persons (Felix et al., 1998; Rebbeck et al., 1998; Wandel et al., 2000); however, studies of the relationship of this SNP to CYP3A4 expression have been inconclusive (Lamba et al., 2002a). Because the pregnane X receptor (PXR)/steroid and xenobiotic receptor is a major regulator of CYP3A-*inducible* expression (Blumberg et al., 1998; Lehmann et al., 1998), the proximal and distal PXR binding elements in *CYP3A4* have also been resequenced but have so far shown no sequence variation (Kuehl et al., 2001; Lamba et al., 2002b).

A consensus is building that human variation in CYP3A4 activity is caused by regulatory polymorphisms rather than structural polymorphisms in the *CYP3A4* gene. The supportive evidence came first from a report that there is significant correlation in human livers between expression of CYP3A4 and CYP3A5 (in polymorphic expressors) (Lin et al., 2002). Further analysis revealed strong correlation between expression of each of the CYP3A family members (CYP3A4, CYP3A5, CYP3A7, and CYP3A43) and between CYP3A4 and PXR (Chang et al., 2003; Westlind-Johnsson et al., 2003). Although at first glance one could conclude that PXR is a likely transcription factor regulating constitutive CYP3A4 expression, mice nullizygous for PXR show either no change, or even a small decrease, in constitutive expression of hepatic CYP3A (Xie et al., 2000; Staudinger et al., 2001). Furthermore, human PXR sequence variants are so rare (Zhang et al., 2001) that they cannot explain variation in CYP3A expression. These findings, coupled with reports of significant correlation between the constitutive androstane receptor (CAR) and CYP3A4, CAR, and PXR (Pascussi et al., 2001) and between CYP2B6, CAR, CYP3A4, and PXR (Chang et al., 2003) in human liver strongly supports the notion that genetic variation in a common hepatic regulatory factor(s) underlies human variability in hepatic CYP3A expression. Thus, the article by Matsumara and colleagues (2004) in this issue of *Molecular Pharmacology* is particularly valuable in their further characterization of transcription factors important for hepatic regulation of CYP3A4.

Matsumara et al. (2004) identify a region between −10.5 and −11.4 kb that functions as a constitutive liver enhancer module (CLEM4). Significantly, this region binds and is reg-

ABBREVIATIONS: kb, kilobase(s); SNP, single nucleotide polymorphism; PXR, pregnane X receptor; CAR, constitutive androstane receptor; CLEM, constitutive liver enhancer module; LCR, locus control region.

ulated by multiple liver-enriched transcription factors, including HNF-1; HNF4; the Jun family member AP-1, which binds to a cAMP response element region; and USF1, which binds to an E-box site. This clustering of transcription regulatory elements is likely to result in a high concentration of transcription factors and strongly supports the notion that this is a site important for CYP3A4 transcriptional activity. Persons with a TGT insertion in the E-box site of the CLEM4 region were identified in the French population. The TGT insertion disrupted binding of USF1 and decreased enhancer activity in in vitro assays.

What is the significance of the TGT insertion to *CYP3A4* promoter activity in vivo? The low allelic frequency in the French population and extremely rare or null frequency among Japanese subjects suggest that it may not contribute significantly to common interindividual variability in CYP3A4 expression. Nevertheless, the study by Matsamura et al. (2004) is useful in its identification of the CLEM and associated binding factors and in identification of additional CYP3A4 promoter variants that may each contribute to variable CYP3A4 expression.

Other liver-enriched transcription factors regulate CYP3A4, including HNF4, HNF3, and CCAAT/enhancer-binding protein-β, and some binding sites in the *CYP3A4* promoter have been identified (Ourlin et al., 1997; Jover et al., 2001; Rodriguez-Antona et al., 2003; Tirona et al., 2003). Clearly, however, much work remains to be done. The *CYP3A4* 5′-flanking region is 35.8 kb (Finta and Zaphiropoulos, 2000) but only ~2.5 kb has been resequenced (Kuehl et al., 2001; Lamba et al., 2002b; this report) and only 13 kb analyzed for hepatic or intestinal regulation (Goodwin et al., 1999; Schuetz et al., 2002; Robertson et al., 2003; this report).

A comparative genomic approach may be useful for pinpointing candidate regulatory motifs (and their putative binding factors) important for CYP3A regulation. Sequence alignment of the intragenic regions from divergent species can identify conserved motifs, what some have called "phylogenetic footprints" (Johnson et al., 2002). Because it is assumed that sequence conservation implies functional importance, these comparative genomic approaches use an evolutionary approach to identify transcriptional regulatory sites. A map of the mouse *CYP3A* locus (Zaphiropoulos, 2003) and phylogenetic analysis of the *CYP3A* gene family among 45 vertebrate species (McArthur et al., 2003) should be helpful in this regard. Indeed, the comparative genomics approach has identified regulatory motifs in a variety of genes, including the globin promoters, that were not identified by biochemical or mutational approaches (Johnson et al., 2002).

It is generally accepted that transcription factors regulating gene expression bind in the 5′ direction to the gene they regulate. There is high sequence conservation between the proximal promoter regions of the *CYP3A* genes, because the *CYP3A* genes arose through a process of gene duplication. The *CYP3A* genes may be coregulated by common transcription factors that bind in the 5′ direction to each *CYP3A* gene. Comparative analysis of the *CYP3A* 5′ regions may reveal additional candidate regulatory regions. For example, is the CLEM4 conserved among the *CYP3A* genes? However, genes in a locus can also share long-range interactions with locus control regions (LCRs). More than 38 mammalian LCRs have been identified (Li et al., 2003); the globin LCR is the most thoroughly described. Although the globin LCR is located 5′ of the globin locus, LCRs can be located upstream, downstream, or within genes that they control. Importantly, some of the first studies on β-globin regulation focused on the proximal 1.5-kb region because it was sufficient to drive β-globin expression in erythroleukemia cells. However, this fragment of DNA was insufficient to support β-globin expression in transgenic mice, because globin expression in erythrocytes requires the LCR, which is located more than 10 kb 5′ of the globin locus (Li et al., 2002). Equally important was that the enhancer activity in the globin LCR was not detectable in transient transfection experiments but could only be detected when the LCR had integrated into the chromatin. This is because LCRs function to maintain open chromatin conformations and enhance transcription. These studies demonstrated that tissue specific regulation of gene expression can depend both on their constitutive expression (regulated by proximal promoters) and tissue specific enhancement of gene expression (governed by LCRs).

One unresolved issue that merits further investigation is whether the *CYP3A* locus has an LCR. Comparative genomics, targeted enhancer trap approaches (Bulger and Groudine, 2002), and YAC transgenic mice (Peterson et al., 1998) harboring various fragments of the *CYP3A* locus will offer powerful approaches to elucidate the promoter regions, enhancers, and possibly an LCR regulating the *CYP3*A genes within the locus. Recently, a transgenic mouse containing the *CYP3A4* gene in a bacterial artificial chromosome was generated. Intriguingly, the *CYP3A4* transgenic mouse expressed CYP3A4 in the intestine but not in the liver (Granvil et al., 2003). Although several explanations are possible, this mouse and other transgenic mice may provide valuable clues on the location of regulatory sequences important for hepatic expression of CYP3A4. Moreover, CYP3A4 is the most highly expressed cytochrome P450 in human intestine and contributes significantly to the first-pass metabolism and decreased oral bioavailability of some substrates (Kolars et al., 1992). Whether the CLEM4 and associated transcription factors identified in the report by Matsumara and colleagues (2004) have any role in regulating CYP3A4 in human enterocytes remains an important question for future experiments. Thus, a major challenge for the future is to determine the transcription factors that singly or together regulate CYP3A4 in liver and intestine. If there is a single regulator that is predictive of hepatic CYP3A, PXR, and CAR expression, then there is still hope for the holy grail, that common genetic variations exist in this important hepatic (and intestinal) regulatory factor(s) and that simple DNA-based tests may predict human variation in CYP3A4 mediated clearance of drugs.

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