Characterisation of *CYP3A* gene subfamily expression in human gastrointestinal tissues

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Abstract

The human CYP3A subfamily is of interest due to its multiplicity, activity toward known carcinogens, and extrahepatic expression. In situ hybridisation analysis of formalin fixed, routinely processed biopsy specimens was used to localise CYP3A mRNA in human gastrointestinal tissues from several individuals. CYP3A mRNA is abundant in human liver and in mucosal epithelial cells of all segments of the human small intestine. RNA blot analyses showed that the mRNA species observed in most livers and in human small intestine represent CYP3A3/3A4 transcripts. This was confirmed at the protein level by immunoblot comparison of small intestine microsomes to in vitro expressed CYP3A4 and CYP3A5 proteins. In liver and small intestine, CYP3A mRNA is not uniformly distributed, with grain density highest in cells within the respective non-proliferative compartments. CYP3A mRNA was also observed in human oesophagus and colon. RNA blot analysis of multiple colons showed heterogeneity in the CYP3A mRNAs present. Two CYP3A mRNAs (CYP3A3/3A4 and CYP3A5) were detected in colon samples from several individuals. In addition to those localisation studies, the capacity of expressed CYP3A4 and CYP3A5 to activate the dietary heterocyclic amine MeIQ in the presence of α -naphthoflavone was shown. These results show that there is considerable heterogeneity in the expression of the CYP3A subfamily in human gastrointestinal tissues.

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Cytochromes P450 represent a superfamily of microsomal heme-thiolate enzymes which catalyse the oxidation of numerous endogenous and foreign compounds.¹⁻³ Individual cytochrome P450 isoforms are characterised by diversity in developmental regulation, substrate specificity, and tissue distribution. While most cytochrome P450 catalysed oxidations result in reduced toxicity of the parent compound, this process occasionally generates a more toxic intermediate. Many such metabolites have been implicated in carcinogenesis. Enzymes encoded by the CYP3A subfamily have been shown to play a pivotal role in the activation of numerous carcinogens including

the polycyclic aromatic hydrocarbon, benzo[a]pyrene,⁴ and the hepatocarcinogen aflatoxin B₁.⁵⁻⁷ Recently, we have also shown that in the presence of dietary flavonoids, members of the CYP3A subfamily are able to activate the heterocyclic amine, 2-amino-3, 4-dimethylimidazo[4,5-f]quinoline (MeIQ), to a mutagen as determined in the Ames/Salmonella test.⁸ This compound represents one of a group of heterocyclic amine compounds formed during the cooking of protein containing foods.^{9,10} Many of these compounds are highly mutagenic in the Ames test, and induce tumours in a number of species at several sites.^{9,10} In the absence of flavonoids, the activation of these compounds is primarily attributed to members of the CYP1A subfamily in both lower animals and humans.¹¹⁻¹⁴

The gastrointestinal tract represents a major portal of entry for many carcinogens. Indeed, Ames *et al* have estimated that more than 90% of the chemicals to which humans are exposed are dietary-derived and must therefore traverse the gastrointestinal tract.¹⁵ Thorough characterisation of the CYP3A subfamily in the human gastrointestinal tract and an accurate assessment of the ability of individual CYP3A enzymes to activate dietary mutagens is therefore essential for a clearer understanding of chemical carcinogenesis in these tissues.

To date, four cDNAs encoding members of the human CYP3A subfamily have been isolated and termed *CYP3A3*, *CYP3A4*, *CYP3A5*, and *CYP3A7*.¹⁶⁻²¹ While the *CYP3A3*, *CYP3A4*, and *CYP3A5* genes are expressed at varying levels in adult human livers,²²⁻²⁴ the *CYP3A7* gene is expressed in fetal liver.^{21,25} Expression of the CYP3A subfamily has also been demonstrated in human extrahepatic tissues, including the gastrointestinal tract.²⁶⁻²⁹ While these studies show CYP3A expression in the human gastrointestinal tract, they include only a small number of individuals and provide conflicting results regarding expression in human colon. In addition, they provide little information regarding which member(s) of the complex CYP3A subfamily are being expressed in gastrointestinal tissues other than the small intestine. We have therefore used a sensitive in situ hybridisation technique complemented with RNA blotting to characterise CYP3A expression at the mRNA level in human gastrointestinal tissues from several subjects. In addition to these localisation studies, we have also determined the ability of two CYP3A subfamily members, CYP3A4 and CYP3A5, to activate two dietary heterocyclic amines in

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the presence and absence of the modulating agent α -naphthoflavone. These studies show that the expression of individual *CYP3A* genes varies considerably throughout the gastrointestinal tract. In addition, considerable interindividual heterogeneity in gastrointestinal *CYP3A* expression is observed in the colon. Given the demonstration of a possible role for *CYP3A* enzymes in the activation of food derived heterocyclic amines in the gastrointestinal tract, such heterogeneity may be important in determining individual susceptibility to chemical toxicity and carcinogenicity.

Methods

MATERIALS AND ENZYMES

All enzymes and reagents required for transcription of RNA probes were obtained from Stratagene (La Jolla, CA). Restriction enzymes and nuclease free bovine serum albumin were obtained from New England Biolabs (MA). [35 S]UTP (1000 Ci/mmol) and α -[32 P]UTP (3000 Ci/mmol) were obtained from Bresatec (Adelaide, Australia). Guanidium thiocyanate, proteinase K, dextran sulphate, diethylpyrocarbonate (DEPC), aminopropyltriethoxysilane (APES), paraformaldehyde, α -naphthoflavone, and NADPH were obtained from Sigma Chemical Co (St Louis, MO). All other chemicals were of analytical reagent grade.

HUMAN TISSUES

Human liver samples were obtained from renal transplant donors maintained on life support systems until the kidneys could be removed. The livers were removed within 30 minutes of death. Human oesophageal, duodenal, jejunal, and colonic tissues were either surgical specimens or needle biopsy sections which had been reported as histologically normal. The use of human tissues in these studies had Flinders Medical Centre Committee of Clinical Investigation approval. Tissues designated for preparation of RNA were snap frozen in liquid nitrogen and stored at -70°C . Tissues for in situ hybridisation or immunohistochemistry were fixed in 10% (v/v) formalin in sodium phosphate buffer (pH 7.4), processed routinely, and embedded in paraffin.

RNA PROBES (RIBOPROBES)

RNA probes were transcribed from a full length *CYP3A4* cDNA of 2059 bases¹⁸ subcloned into the Bluescript SK transcription plasmid (Stratagene, CA). Due to the high degree of sequence similarity within the *CYP3A* subfamily, the *CYP3A4* RNA probe would be expected to detect transcripts encoding all *CYP3A* subfamily members. The recombinant plasmids were linearised and subjected to proteinase K digestion before RNA probe synthesis. RNA probes were reduced in size by limited alkaline hydrolysis. Both anti-sense and sense riboprobes were transcribed from the *CYP3A4* cDNA for use as test and control probes respectively. RNA

probes were labelled by incorporation of [35 S]UTP for in situ hybridisation studies or α -[32 P]UTP for RNA blots.

BLOT ANALYSIS OF RNA

Total RNA was isolated from human liver by the guanidium method of Chomczynski.³⁰ Purification of mRNA was by repeated oligo(dT)-cellulose chromatography.³¹ All RNA preparations were assessed for integrity by electrophoresis and staining with ethidium bromide before use. RNA blot analyses were carried out by electrophoresis of mRNA aliquots on 1% agarose-2.2 M formaldehyde gels,³² blotting to nylon membrane (Amersham, Australia) using a Posiblot pressure blotter (Stratagene, La Jolla, CA), cross linking of mRNA to membrane using a UV Stratalinker 1800 (Stratagene, La Jolla, CA) and hybridisation with 32 P-labelled *CYP3A4* RNA probes. Hybridisation was performed at 50°C in 50% formamide, $5\times\text{SSC}$ ($1\times\text{SSC}=0.15\text{ M}$ sodium chloride and 15 mM sodium citrate), $1\times\text{PE}$ ($1\times\text{PE}=50\text{ mM}$ Tris-HCl pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.2% ficoll, 0.2% polyvinylpyrrolidone, 5 mM EDTA) and $150\text{ }\mu\text{g/ml}$ denatured salmon sperm DNA. Unbound probe was removed by washing in $0.1\times\text{SSC}$ at 62.5°C . Bound probe was visualised by autoradiography using an intensifying screen (Cronex, DuPont).

IN SITU HYBRIDISATION

The in situ hybridisation procedure was performed using $4\text{ }\mu\text{m}$ formalin fixed, paraffin embedded sections as described previously.³³ Two serial sections were included on each slide for probing with test (anti-sense) and control (sense) probes respectively. A high stringency wash was necessary to reduce background with riboprobes and was performed in $0.1\times\text{SSC}$ containing 30% formamide at 62.5°C . When necessary, background was further reduced by a 15 minute incubation with $2\text{ }\mu\text{g/ml}$ RNase A (Promega, Sydney, Australia) in $2\times\text{SSC}$ at 37°C .

Figure 1: RNA blot analysis of human liver mRNA samples. The alternative transcript *CYP3A3/CYP3A4* mRNA sizes are 2.2 and 3.0 kb.²⁰ The *CYP3A5* mRNA is approximately 1.9 kb. The *CYP3A4* RNA probe is not specific and recognises all *CYP3A* subfamily members. Lanes 1-5 contain $5\text{ }\mu\text{g}$ of human liver mRNA. (Hybridisation at 50°C ; wash at 65°C in $0.1\times\text{SSC}$.)

Figure 2: In situ hybridisation – liver. (A) Negative control section of human liver (H35) probed with ^{35}S -labelled CYP3A4 sense riboprobe; (B) test section of human liver probed with the ^{35}S -labelled CYP3A4 antisense probe showing increased numbers of autoradiographic grains relative to the control section with grain density highest in hepatocytes surrounding the terminal hepatic venule; and (C) alternative area of test section shown in (B) under dark field illumination (haematoxylin and eosin, original magnification $\times 100$).

EXPRESSION OF CYP3A4 AND CYP3A5

The human CYP3A4 and CYP3A5 cDNAs were isolated as previously described.^{18,19} The cDNAs were subcloned into the COS cell expression vector p91023(B). COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% Nu-serum. Transient transfections were performed as described by Zuber *et al.*³⁴ Briefly, DNA (10 $\mu\text{g}/\text{ml}$) was transfected onto COS-7 cells for one hour using the DEAE-dextran method, followed by a five hour incubation with chloroquine (40 $\mu\text{g}/\text{ml}$). Cells were isolated 72 hours after transfection, resuspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, and stored at -70°C until used. As a control, cells were also transfected with the p91023(B) vector lacking a CYP3A insert.

POLYACRYLAMIDE GEL ELECTROPHORESIS AND IMMUNOBLOTTING

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (0.1%) was performed essentially as described by Laemmli.³⁵ Immunoblotting was performed as described previously.^{11,12} Blots were developed with a polyclonal goat anti-human CYP3A3 IgG which has been previously characterised.¹¹ This antibody recognises purified human CYP3A3 and also recognises COS cell expressed CYP3A4 and CYP3A5. Transfer of proteins from polyacrylamide gels to nitrocellulose paper was accomplished in 16 hours at 30 mA in a 'Trans-blot Cell' (Bio-Rad, Sydney, Australia).

AMES TEST

The mutagenicity of the heterocyclic amine MeIQ was tested on *Salmonella typhimurium* TA98 by the method of Maron and Ames,³⁶ incorporating a 20 minute incubation step

before pouring the plates. A detailed account of the methodology used in these experiments has recently been published.³⁷

Results

LOCALISATION OF CYP3A mRNA IN HUMAN LIVER

As outlined in the methods section, RNA probes for in situ hybridisation analysis of CYP3A mRNA in human tissues were prepared by limited alkaline hydrolysis of transcripts from a full length CYP3A4 cDNA. To assess the specificity of the hydrolysed CYP3A4 RNA probe for CYP3A subfamily members and its suitability for in situ hybridisation analysis of CYP3A mRNA in human tissues, a series of blot hybridisation analyses of human liver mRNA was performed. Equal aliquots of hepatic mRNA (5 μg) from 18 human livers in total were analysed with the CYP3A4 riboprobe. Probing of human liver mRNA blots with the CYP3A4 RNA probe demonstrated the existence of two distinct CYP3A expression profiles. A representative blot of several liver mRNA samples clearly demonstrating these mRNA patterns are shown in Figure 1. Most livers demonstrated

Figure 3: RNA blot analysis of human small intestine mRNA samples. CYP3A3/3A4 mRNA sizes are 2.2 and 3.0 kb. Lanes contain 10 μg mRNA from human (I) ileum and (J) jejunum. (Hybridisation at 50°C ; wash at 65°C in $0.1\times\text{SSC}$.)

Figure 4: RNA blot analysis of human gastrointestinal mRNA samples. The alternative transcript CYP3A3/CYP3A4 mRNA sizes are 2.2 and 3.0 kb. The CYP3A5 mRNA is approximately 1.9 kb. Lanes 1 to 3 contain 5 µg of human liver mRNA, lane 4 contains 5 µg jejunum mRNA, lanes 5 to 15 contain 10 µg of colon mRNA. (Hybridisation at 50°C; wash at 65°C in 0.1×SSC.)

the existence of a two band (2.2 kb and 3.0 kb) pattern. It has previously been shown that these two mRNA species represent separate transcripts encoding either CYP3A3 or CYP3A4 and arise because of the use of two distinct polyadenylation signals.²⁰ As a result of the close relationship between the CYP3A3 and CYP3A4 genes, their relative expression has not been adequately characterised at either the mRNA or protein level. In two of the 18 livers studied, a third mRNA species (approximately 1.9 kb) was observed. The lower molecular weight of this mRNA is consistent with the 1708 bp CYP3A5 cDNA as compared with the larger CYP3A3 and CYP3A4 cDNAs.¹⁹ This result is also consistent with the expression of the CYP3A5 enzyme in addition to CYP3A3/3A4 in these two livers as assessed by immunoblotting of microsomes with anti-human CYP3A3 IgG (R A McKinnon, W M Burgess, and M E McManus, unpublished results).

In situ hybridisation analysis of CYP3A mRNA in human liver was performed on formalin fixed, paraffin embedded sections prepared from several livers using the hydro-

lysed CYP3A4 antisense RNA probe. All test sections of liver were evaluated by direct comparison with a control section probed with a CYP3A4 sense RNA probe. A typical negative control section of normal liver is shown in Figure 2(A). Control sections showed few autoradiographic grains which were uniformly distributed. This signal was interpreted as background. In contrast, sections probed with the test CYP3A4 (antisense) RNA probe showed grain densities appreciably greater than those seen in control sections and this was interpreted as a positive result. Representative sections of human liver probed with the CYP3A4 antisense RNA probe are shown under conventional and dark field illumination in Figure 2, (B) and (C) respectively. Autoradiographic grains were distributed non-uniformly across the liver acinus with grain density highest in perivenular (zone 3) hepatocytes, extending into zone 2. The demarcation between hepatocytes that showed high levels of autoradiographic grains and those with signal only marginally above background was particularly sharp in sections probed with the CYP3A4 probe. No increase in autoradiographic grain levels was seen in liver cells other than hepatocytes.

EXPRESSION OF THE CYP3A SUBFAMILY IN THE HUMAN GASTROINTESTINAL TRACT

The CYP3A4 RNA probe was used to probe RNA blots of mRNA samples prepared from surgical specimens of human jejunum, ileum, and colon. RNA blot analysis of jejunal and ileal mRNA samples showed 2.2 kb and 3.0 kb bands as observed in most human liver mRNA samples (Fig 3). This result shows that the expression of the CYP3A subfamily in human small intestine mimics that observed in the majority of human livers, with CYP3A3/3A4 representing the major form(s) of CYP3A mRNA present. The 1.9 kb band observed in two human livers was not observed in the small intestine mRNA samples. RNA blot analysis of mRNA samples from 11 human colons showed considerable heterogeneity in CYP3A mRNA with at least two genes expressed in some colons and none in others (Fig 4). A 2.2 kb band representing the predominant CYP3A3/3A4 transcript was observed in five of the 11 samples. The level of this mRNA species was considerably lower in all colon specimens than in small intestine or liver. A 1.9 kb band similar in mobility to that observed in two liver samples known to express CYP3A5 was observed in six of 11 individuals. This heterogeneity in CYP3A expression did not seem to correlate with sex or the region of colon analysed (Table I). The 3.0 kb band observed in liver was not observed in any sample of colonic mRNA. Degradation of mRNA samples is an unlikely explanation for the observed differences as ethidium bromide staining of gels before transfer showed minimal RNA degradation. In addition, laser densitometry of these blots before final washing at high stringency demonstrated only a twofold variation in the intensity of the 28S and 18S

TABLE I Expression of CYP3A genes in human colon by RNA blotting

Colon	Sex	Age (y)	Region	CYP3A3/4	CYP3A5
C5	F	73	Ascending	—	+
C7	F	56	Sigmoid	—	—
C9	F	44	Ascending	—	+
C10	M	63	Rectum	+	+
C11	M	55	Ascending	—	—
C14	M	73	Sigmoid	+	—
C15	M	72	Sigmoid	+	+
C16	F	85	Ascending	—	—
C24	M	70	Descending	—	—
C17	M	70	Ascending	+	+
C _R	M	64	Unknown	+	+

Figure 5: Immunoblot analysis of ileal and jejunal microsomes with anti-human CYP3A3 IgG. Electrophoresis was performed as described in the methods section. Migration of proteins is from the top to the bottom of the gel. Lanes contain: H11 and H12, human liver microsomes (10 µg); I, human ileum microsomes (25 µg); J, human jejunum microsomes (25 µg); CYP3A4 and CYP3A5; 100 µg cell lysate protein from transfected COS-7 cells expressing CYP3A4 and CYP3A5 respectively. (Anti-human CYP3A3 IgG 1:2000.)

ribosomal RNA bands. We are therefore confident that the amounts of RNA loaded per lane are within twofold of one another.

Immunoblot analysis was also performed on human gastrointestinal tissues using a polyclonal anti-human CYP3A3 IgG to assess CYP3A expression at the protein level. As discussed, this antibody recognises up to two bands in human liver corresponding to CYP3A3/3A4 and CYP3A5. Immunoblotting in our laboratory has shown the CYP3A3/3A4 band in all livers studied while the CYP3A5 band has been observed in five of 22 individuals (R A McKinnon, W M Burgess, and M E McManus, unpublished results). Immunoblotting was performed on microsomes prepared from human stomach, jejunum, ileum, and colon. While a single immunoreactive band was observed in human ileum and jejunum, no immunoreactivity was observed in stomach or colon microsomes. To determine the identification of the immunoreactive species in the small intestinal microsomes, cell lysate preparations of COS-7 cells in which either CYP3A4 or CYP3A5

protein had been expressed were included on these immunoblots as standards. A representative immunoblot is shown in Figure 5. The single band observed in microsomes prepared from human ileum and jejunum co-migrated with both the CYP3A3/3A4 band routinely observed in all human livers and the cDNA-expressed CYP3A4 protein band. As CYP3A3 and CYP3A4 cannot be electrophoretically separated using SDS-PAGE, these data show that one or both of these enzymes, rather than CYP3A5, represents a major constitutive enzyme in the human small intestine.

The CYP3A4 RNA probe was used to localise CYP3A mRNA in formalin fixed, paraffin embedded sections from various segments of the human gastrointestinal tract using in situ hybridisation. All test sections were evaluated by direct comparison with a control section probed with a CYP3A4 sense RNA probe. In situ hybridisation analyses were also correlated with immunohistochemical studies using an anti-CYP3A3 polyclonal IgG for further verification. Autoradiographic grains indicating the presence of CYP3A mRNA were observed on sections of human oesophagus, small intestine, and colon but not on sections of human stomach. The level of signal was highest in the small intestine. Analysis of sections of oesophagus from four individuals showed CYP3A mRNA in all cases. The level of signal was variable but was lower than observed in the small intestine. Figure 6(A) shows a representative section of human oesophagus probed with the CYP3A4 sense probe while a serial section probed with the CYP3A4 antisense probe is shown under conventional and dark field illumination in Figure 6, (B) and (C) respectively. As expected from the RNA blot results, appreciable levels of autoradiographic grains were observed on sections prepared from all segments of the human small intestine. Autoradiographic grains were localised to the surface columnar absorptive cells throughout the human small intestine with grain density highest at the tips of the villi. In contrast, few grains were observed in Goblet cells and the epithelial cells of the crypts of Lieberkuhn. Brunner's glands also showed grain levels not appreciably higher than background. Some interindividual variation was observed in the level of autoradiographic signal on sections, particularly in the jejunum. A representative control section of a duodenal biopsy probed with a CYP3A4 sense RNA probe is shown in Figure 7(A). A test section probed with the antisense RNA probe, clearly showing CYP3A mRNA, is shown under both conventional illumination and dark field illumination in Figure 7, (B) and (C) respectively.

Whereas an abundance of autoradiographic signal was observed on analysis of all human small intestine sections, analysis of colon sections from several individuals produced more variable results. Autoradiographic signal indicating the presence of CYP3A mRNA was localised to mucosal epithelial cells on colonic biopsy sections from eight of 10 individuals studied. The level of autoradiographic signal

Figure 6: In situ hybridisation - oesophagus. (A) Negative control section of oesophageal biopsy specimen probed with ^{35}S -labelled CYP3A4 sense riboprobe; (B) test section of oesophageal biopsy specimen probed with ^{35}S -labelled CYP3A4 antisense probe; and (C) section (B) shown under dark field illumination. (Haematoxylin and eosin, original magnification $\times 200$.)

Figure 7: In situ hybridisation – duodenum. (A) Negative control section of duodenal biopsy specimen probed with ^{35}S -labelled CYP3A4 sense riboprobe; (B) test section of duodenal biopsy specimen probed with ^{35}S -labelled CYP3A4 antisense probe with autoradiographic gains localised to the surface epithelial cells; and (C) section (B) shown under dark field illumination. (Haematoxylin and eosin, original magnification $\times 100$.)

observed on these sections was extremely variable but was reduced relative to that routinely observed on sections of small intestine. A representative control colon biopsy section probed with the CYP3A4 sense RNA probe is shown in Figure 8(A). A colonic section probed with the CYP3A4 antisense RNA probe is shown under conventional and dark field illumination in Figure 8, (B) and (C) respectively. The level of autoradiographic signal was variable between colon sections obtained from different individuals. Analyses of multiple segments of the colon from single individuals showed that when present, CYP3A mRNA was expressed in all segments of the colon (caecum, ascending, transverse, descending, sigmoid) studied. Autoradiographic signal was also observed in rectum but at levels lower than elsewhere in the colon.

Figure 8: In situ hybridisation – colon. (A) Negative control section of colon biopsy probed with ^{35}S -labelled CYP3A4 sense riboprobe; (B) test section of colon biopsy tissue probed with ^{35}S -labelled CYP3A4 antisense probe; and (C) section (B) shown under dark field illumination. (Haematoxylin and eosin, original magnification $\times 200$.)

ACTIVATION OF DIETARY HETEROCYCLIC AMINES BY EXPRESSED CYP3A4 AND CYP3A5

To pursue the possibility that CYP3A enzymes, when expressed in gastrointestinal tissues, may play a role in dietary promutagen activation, human CYP3A4 and CYP3A5 were expressed in a COS cell expression system as described in the methods section. Immunodetection of the COS cell-expressed CYP3A proteins by goat anti-human CYP3A3 IgG is evident from the immunoblot featured in Figure 5. The activity of CYP3A4 and CYP3A5 was then determined toward two dietary heterocyclic amines, PhIP and MeIQ, in the Ames *Salmonella* test. Neither CYP3A enzyme exhibited activity toward PhIP in the absence or presence of 100 μM α -naphthoflavone (data not shown). The capacity of CYP3A4 and CYP3A5 to activate the heterocyclic amine MeIQ is shown in Table II. In the absence of α -naphthoflavone, both CYP3A4 and CYP3A5 possessed minimal activity toward MeIQ. This activity increased 16 fold (CYP3A4) and 10 fold (CYP3A5) with the addition of 100 μM α -naphthoflavone. No activity towards PhIP or MeIQ was observed in cell lysates prepared from COS cells transfected with the expression vector p91023(B) lacking a CYP3A insert.

Discussion

We have used a variety of techniques to study the expression of the CYP3A subfamily in the human gastrointestinal tract. Using the in situ hybridisation technique, CYP3A expression was shown in human liver, oesophagus, duodenum, jejunum, ileum, and all segments of the colon. The zonal pattern of CYP3A mRNA localisation in human liver and all areas of the small intestine correlated with the immunohistochemical findings of Murray *et al*^{28 38} indicating that the previously observed zonal distribution of CYP3A protein in these tissues is secondary to differences in mRNA levels. In all human liver sections, autoradiographic grains were localised in a noticeably non-uniform pattern, maximal in perivenular hepatocytes. Several earlier studies have also reported a predominantly perivenular distribution of individual cytochrome P450 enzymes, both in rats^{39–43} and humans.^{33 44} In addition, a similar distribution has been reported for the human NADPH-cytochrome P450 reductase, the requirement for which is common to all forms of cytochrome P450 other than certain liver mitochondrial forms.⁴⁵ A 'zonal' distribution of CYP3A mRNA was also evident in the small intestine with the CYP3A mRNA maximal at the villus tip. The lack of uniformity in CYP3A mRNA in both liver and small intestine is consistent, the expression of CYP3A genes being minimal in the proliferative compartment of the respective tissues and maximal in cells residing in the non-proliferative compartment. The exact mechanism(s) underlying zonal heterogeneity in P450 expression is presently unclear although the results in this study clearly indicate that zonal differences arise as a result

TABLE II Mutagenic activation of MeIQ (1 µg) by expressed CYP3A4 and CYP3A5 in the absence and presence of 100 µM α-naphthoflavone (values, mean (SD))

Expressed P450	Mutagenicity (# revertants/mg protein)	
	Control	α-naphthoflavone
CYP3A4	360 (311)	5913 (428)
CYP3A5	446 (544)	4780 (612)

n=3.

of altered transcription of genes or mRNA stabilisation rather than translational or post-translational mechanisms. A possible factor governing heterogeneity in gene transcription may be the varied circulatory microenvironment of cells within different zones and therefore varied exposure to circulating agents such as hormones or xenobiotic inducers. While such a mechanism may partly explain the zonal regulation of some P450s, the abrupt acinar demarcation in the hepatic expression of CYP3A genes shown in the present studies suggests that other mechanisms must also be involved. While the mechanism underlying such zonal specificity in the liver remains unclear, the increased levels of cytochrome P450 expression and subsequent raised rates of toxic metabolite formation in perivenular hepatocytes may be a significant contributing factor to the zonal nature of chemical induced liver injury.⁴⁶

The present studies show that there is considerable variation in CYP3A gene expression in the gastrointestinal tract, with the small intestine and colon demonstrating distinct CYP3A complements. From both RNA and immunoblot studies it seems that the expression of CYP3A genes in the small intestine is limited to CYP3A3/3A4 expression, although it is possible that screening of a larger number of individuals may demonstrate some CYP3A5 expression. The present findings in the small intestine are consistent with the recent demonstration of CYP3A4 expression in the small intestine using form-specific oligonucleotide probes.²⁹ The expression of CYP3A genes in the human colon was distinct from that in the small intestine and showed considerable interindividual heterogeneity. The finding of an mRNA apparently corresponding in size to CYP3A5 in a greater number of individuals than CYP3A3/3A4 mRNA was of particular interest given the contrary findings in liver. While no CYP3A mRNA was observed in a number of individuals using either in situ hybridisation or RNA blotting, this finding was not unexpected given that in a previous study we were unable to detect CYP3A protein in human colon using the immunoblot technique.⁸ In addition, Murray *et al* were unable to show CYP3A immunoreactivity in the human colon,²⁸ although other groups have demonstrated CYP3A immunoreactive protein in microsomes prepared from human colon.^{27 47 48} The identification of colonic CYP3A expression in a large number of subjects in this study probably reflects the excellent sensitivity of both the in situ hybridisation and RNA blotting techniques employing

RNA probes. The demonstration of CYP3A expression by both techniques required long autoradiographic exposures indicating the relatively low level of expression in this tissue. The interindividual heterogeneity in CYP3A expression observed in the colon did not correlate with gender or the anatomical location within the colon. Indeed, in individuals in whom CYP3A mRNA was observed in colon by RNA blotting, in situ hybridisation analysis of segments of caecum, ascending colon, transverse colon, descending colon, and sigmoid colon confirmed expression along the whole length of the colon. While the heterogeneity in colonic CYP3A mRNA may reflect genetic variations in CYP3A expression, these differences may be a result of differential induction of the various CYP3A enzymes. While drug histories obtained for these individuals were incomplete, there was no evidence that any subject was undergoing therapy with known inducers of the CYP3A subfamily.

In a previous study we showed that a potential interaction involving CYP3A enzymes may exist between the dietary heterocyclic amines and flavonoids consumed in the diet.⁸ This earlier study demonstrated the capacity of microsomes prepared from rabbit and human small intestine to activate the heterocyclic amine MeIQ in the presence of flavonoids. We postulated that such an interaction may be due to activation of MeIQ by CYP3A enzymes. Confirmation of this hypothesis and its potential importance in humans required both the further characterisation of gastrointestinal CYP3A expression, as described in the present report, and a demonstration that members of the CYP3A subfamily were capable of carrying out this process. To explore this latter possibility we expressed two members of the CYP3A subfamily from their respective cDNAs and determined their activity toward two dietary heterocyclic amines, PhIP and MeIQ, in the presence and absence of the flavonoid α-naphthoflavone. These two compounds were selected as PhIP represents the most abundant of the heterocyclic amine compounds found in cooked meat while MeIQ exhibits far greater mutagenicity.^{9 10} In the present study we have shown that the capacity of both CYP3A4 and CYP3A5 to activate MeIQ to a mutagen is increased significantly in the presence of α-naphthoflavone. In contrast, no activity was observed for either CYP3A4 or CYP3A5 towards PhIP. The demonstration that expressed CYP3A4 and CYP3A5 proteins are indeed able to activate MeIQ to a mutagen in the presence of α-naphthoflavone supports the hypothesis that CYP3A proteins are responsible for the flavonoid inducible MeIQ activation observed with small intestine microsomes.⁸ The significance of such an interaction in the aetiology of human cancer is unclear, however, given that most human gastrointestinal cancers occur in the colorectal region yet CYP3A expression is greater in the small intestine.

This study again shows the utility of the in situ hybridisation technique for investigating

cytochrome P450 expression in human tissues. The analysis of cytochrome P450 expression in human tissues is often difficult because of the multiplicity of cytochromes P450 and limited availability of human tissues. In situ hybridisation, when complemented with RNA blotting studies, obviates many of these difficulties and is therefore particularly suited to analysis of cytochrome P450 expression. The technique provides precise spatial information not possible with conventional blotting techniques and also offers excellent sensitivity. We have previously shown the suitability of the in situ hybridisation technique for localising cytochrome P450 mRNA in routinely processed formalin fixed, paraffin embedded tissue sections.³³ This enables retrospective studies of P450 expression in a range of both normal and pathological tissues. The ability to use routine biopsy specimens is particularly useful for studies of gastrointestinal expression given the frequency of biopsy procedures in these tissues. The present study shows many of the advantages of in situ hybridisation enabling a broader study of gastrointestinal CYP3A expression. This has allowed the detection of low level CYP3A expression in colonic tissue and demonstration of the heterogeneity in such expression within tissues and between individuals. While the present study clearly shows the application of in situ hybridisation for the precise and sensitive localisation of CYP3A expression in human tissues, complete characterisation of the CYP3A subfamily requires further analysis with probes able to differentiate the highly related CYP3A3 and CYP3A4 sequences. The high sequence similarity of these two P450s will necessitate the use of oligonucleotide probes for differentiation. Oligonucleotide probes have been successfully employed for in situ hybridisation analysis of cytochrome P450 mRNA in rat tissues³⁹ and extension of such studies to human tissues will enable definitive characterisation of intestinal P450 expression and a clearer insight into possible mechanisms of chemical carcinogenesis.

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