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Interindividual Differences in Hepatic Expression of CYP3A4: Relationship to Genetic Polymorphism in the 5′-Upstream Regulatory Region

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Cytochrome P450 3A4, the most abundant P450 form in human liver, exhibits a very broad substrate specificity and is of great importance for drug metabolism. The interindividual difference in the hepatic expression of CYP3A4 is considerable. In order to investigate possible genetic factor(s) causing this variation, the rate of 6β-hydroxylation of testosterone in human liver microsomes prepared from 46 different human liver samples was determined and the 5′upstream region (+10 to −490 bp) was sequenced from genomic DNA isolated from 39 of these livers. We found a 31-fold variation of the testosterone hydroxylase activity between the samples. However, a very high sequence homology between the *CYP3A4* 5′-upstream regions sequenced from the 78 different alleles was found. In fact, only three variant nucleotide exchanges were identified, all causing a −290 A→G mutation (CYP3A4-V) in a so called nifedipine specific element (NFSE). The importance of this element and the polymorphism was evaluated by gel shift analysis. Competition experiments revealed that the binding of nuclear proteins, although having lower affinity to the CYP3A4-V form of the element, was unspecific in nature. In accordance, no influence of this polymorphism was seen on the microsomal testosterone hydroxylase activity *in vitro*. It is concluded that the promoter region of *CYP3A4* is highly conserved, the only polymorphism being in the NFSE, which however does not influence the enzyme expression in liver to a significant degree. This casts doubt of a previously described relationship between the *CYP3A4-V* allele and cancer in the prostate and leukaemia. © 1999 Academic Press

Key Words: **nifedipine; testosterone hydroxylase; pregnenolone X receptor; leukaemia; prostate cancer.**

Cytochrome P450 3A4 (CYP3A4) is the major form of P450 in human liver, accounting for 30% of total CYP protein content (1). CYP3A4 exhibits a broad substrate specificity and is apparently involved in the metabolism of over 50% of all drugs used in humans (2). There is a large interindividual variability in the expression of CYP3A4, up to 40-fold (3). The origin of this variation is so far unknown but may be caused by genetic, environmental, pathological, hormonal and dietary factors. An explanation put forward is genetic variation within regions important for transcriptional control. In a recent study, a transition mutation (−290 A→G) was reported which disrupts a so called nifedipine-specific element (NFSE) at −287 to –296 bp in the 5′regulatory region of the gene (4). The allele frequency of this variant allele, termed the *CYP3A4-V*, was estimated to be 9% in Caucasians, 53% in African Americans and 0% in a Taiwanese population, hence this polymorphism exhibit considerable interethnic variability (5). Rebbeck et al. reported that the *CYP3A4-V* allele was associated with tumours of higher clinical stage and grade in men with prostate cancer. The same group suggested that individuals carrying the *CYP3A4-V* allele have lower expression of CYP3A4, decreased testosterone 6β-hydroxylase activities and thus increased testosterone levels which affect androgen-mediated prostate carcinogenesis (4). The *CYP3A4-V* genotype was also shown to be underrepresented in patients with treatment related leukaemia (6). Also these authors suggested this allele to be associated with decreased expression of the enzyme and therefore carriers of this allele would have decreased capacity for activation of precarcinogens activated by CYP3A4.

Nuclear proteins have been shown to bind to NFSE, but their affinity for this element has not been investigated (7). Neither has it been considered whether this element is involved in the transcriptional regulation of the *CYP3A4* gene even though it is located in a region

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Abbreviations used: CYP, Cytochrome P450; bp, base pair(s).

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of potential interest with neighbouring elements binding the newly identified receptors, hPXR (8) and SXR (9) as well as the glucocorticoid receptor.[2]

In order to investigate the presence of any genetic polymorphism in the *CYP3A4* promotor and the most proximal 5′-upstream region, the region +10 to −490 bp of the *CYP3A4* gene was sequenced using genomic DNA from 39 different individuals and the distribution of variant alleles correlated to the hepatic *in vitro* activity of testosterone 6β-hydroxylation, a reaction mainly catalysed by CYP3A4 (10, 11). The results indicate a high extent of sequence conservation in this area of the *CYP3A4* gene but a pronounced interindividual variation in CYP3A4 expression.

MATERIALS AND METHODS

Chemicals. Testosterone was obtained from Sigma (St. Louis, MO). 4-Androstene-3,17-dione and 6β-hydroxytestosterone were purchased from Steraloids Inc. (Newport, RI).

Human liver microsomes. A panel of liver pieces from 46 different individuals was established. Eighteen samples (HL 109–HL 129), originated from patients undergoing liver resections, in most cases due to malignant tumours (usually metastatic colon cancer) and was obtained from Sahlgrenska Hospital, Gothenburg, Sweden. The liver samples were collected from portions of the liver without pathological changes. Twenty-eight liver samples (HL 16–HL 55) were obtained from organ donors who met accidental deaths (Table I). Procedures for isolation of liver pieces have previously been described by Ekström et al. (12). The study was approved by the ethical committee at Karolinska Institutet, Stockholm.

Microsomes were prepared by subcellular fractionation as described elsewhere (13). The frozen liver samples were allowed to thaw at 4°C in five volumes of homogenising buffer (250 mM sucrose, 50 mM Tris-HCl, pH 7.4). The human liver microsomes were suspended in 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and pearl frozen in liquid nitrogen and stored at −70°C. The total protein content was determined by the method of Lowry (14).

Enzyme assay. Human liver microsomes (0.25 mg) were incubated at 37°C for 15 min in 0.5 ml reaction mixtures containing potassium phosphate (50 mM, pH 7.4), $MgCl_2$ (30 mM) in the presence of 200 μM testosterone (added in 5 μl ethanol). The reactions were initiated with NADPH (1 mM) and terminated by removal to ice and addition of 2.5 ml of ethyl acetate. Each sample was spiked with an internal standard (48 nmol 4-androstene-3,17-dione). Zero time incubations served as blanks and blanks spiked with 6β-hydroxytestosterone (22.3 nmol in 20 μl ethanol) served as external standards. After extraction, centrifugation and separation, the organic phase was removed and evaporated under nitrogen. All incubations were performed at conditions under which linearity with time was established.

HPLC analysis. The residues from evaporation were dissolved in 200 μl of mobile phase. Aliqouts of 100 μl were injected onto the HPLC. Testosterone metabolites were resolved on a reversed phase Zorbax C_{18} column (5 μm, 150 × 4 mm) and eluted with binary gradient of mobile phase A and B. Mobile phase A was a 20:80 (v/v) mixture of acetonitril:H_2O and mobile phase B was a 60:40 (v/v) mixture of the same components. A concave gradient from 100% A to 100% B was operated over 37 min at ambient temperature, followed by 5 min re-equilibration with 100% mobile phase A, at the flow rate 0.8 ml/min. Testosterone and its potential metabolites were monitored at 254 nm and quantified by comparison of their peak areas with those of standards and the approximate retention times for 6β-testosterone, testosterone and 4-androstene-3,17-dione were 14.5, 28.3 and 29.6 min respectively.

DNA isolation and sequencing. Genomic DNA was isolated from 39 human liver tissue samples using a QIAamp tissue kit (QIAGEN). *CYP3A4* specific primers 5′-CTGCAGTGACCACTGCCCCA-3′ and 5′-CTTTGCTGGGCTATGTGCA-3′, were used for gene specific amplification of the 5′-flanking region (+46 to −1105) of the *CYP3A4* gene (Genbank D11131), from 30-80 ng of genomic DNA. The resulting fragment served as a template for a second PCR amplifying the specific region of interest (+46 to −505 bp) for sequencing using the forward primer 5′-AAAGTGCCCAGGGGAGG-3′ and the reverse primer 5′-CTTTGCTGGGCTATGTGCA-3′, the latter also used in the sequencing reaction. The purified (Wizard kit, PROMEGA) PCR product (20-80 ng) was used in cycle sequencing reaction using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer). The precipitated product was analysed on a ABI PRISM 377 DNA sequencer (Perkin Elmer) with a ready readable sequence from +10 to −490 bp as a consistent result.

Gel mobility shift assay. Nuclear extracts from human liver tissue were prepared according to the protocol described by Nakabayashi et al. (15). A 22 bp synthetic oligonucleotide NFSE, 5′-AGAGACAAGGGCA**A**GAGAGAGG-3′ corresponding to the NFSE element were used as probe. Unlabeled NFSE oligo and unlabeled oligo corresponding to the mutated variant NFSE-V, 5′-AGAGACAAGGGCA**G**GAGAGAGG-3′, were used in competition experiments. 20 μg extracts, 15 fmol of ^{32}P labelled oligonucleotide and 2 μg poly (dI · dC):(dI · dC) were added to the mixture containing 28 mM Hepes (pH 7.9), 50 mM or 120 mM KCl, 1.5 mM NaCl, 0.4 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 15.5% Glycerol in a final volume of 20 μl and incubated at 25°C for 60 min. For competition experiments indicated, 20, 50 and 2000 fold molar excess unlabeled oligonucleotide was used. DNA-protein complexes were separated under nondenaturing conditions in a 4% polyacrylamide gel (37.5:1) at 4°C as previously described (16).

RESULTS

The hepatic microsomal testosterone 6β-hydroxylase activities in 46 liver samples were measured and found to differ by a factor of 31, ranging from 310 pmol/mg/min to 9560 pmol/mg/min (Table I). The mean level ± SD of testosterone 6β-hydroxylase activity was 2220 ± 2050 pmol/mg/min. No significant differences was seen between samples from male or female subjects.

The promoter region area (+10 to −490 bp) of the *CYP3A4* gene was sequenced. Results from 78 different alleles showed a very high sequence homology. Only three variant alleles were found and they contained a −290 A→G mutation. Thus, three subjects were heterozygous for this allele, yielding an allele frequency of 3.8%. Examination of the rate of testosterone 6β-hydroxylation activity in liver microsomes from these three individuals revealed that two samples had medium rate of activity (2380 pmol/mg/min and 2610 pmol/mg/min whereas the third sample exhibited extraordinary high activity, 9560 pmol/mg/min. However this individual had taken barbiturates before resection, compounds known to be potent inducers of CYP3A4 (17, 18).

[2] Abstract No. 151 at 12th International Symposium on Microsomes and Drug Oxidation 1998, by Pacussi, J. M., Calleja, C., Mani, J. C., Barret, C., Jourdaidi, Y., Maurel, P., and Vilarem, M. J.

TABLE I

List of Human Liver Samples

Sample No.	Sex	Age	Known alcohol, cigarette or drug intake	Rate of 6β-hydroxylation of testosterone in pmol/mg/min[d]	Genotype (wt/wt[a], wt/mut[b], or mut/mut[c])
HL 16	M	51	alcohol, oxazepam, propanolol, promethazin, propiomazin, melperon, orphenadine	3660	n.d.*
HL 18	F	59	smoker	3070	n.d.*
HL 19	M	13	—	1420	wt/wt
HL 20	F	56	barbiturates	9560	wt/**mut**
HL 21	M	65	smoker, glucocorticoids, phenytoin	5060	wt/wt
HL 25	F	52	diazepam, promethazine	2000	wt/wt
HL 28	F	44	—	5370	wt/wt
HL 29	F	62	smoker	7630	n.d.*
HL 32	F	65	—	1850	wt/wt
HL 33	F	25	—	810	n.d.*
HL 35	M	56	hydrochlorthiazide, hydralizine, glucocorticoids	2120	wt/wt
HL 36	M	21	—	9080	n.d.*
HL 38	F	38	—	580	wt/wt
HL 39	F	62	—	1360	wt/wt
HL 41	M	34	pentobarbital	370	wt/wt
HL 42	M	31	phenytoin	2650	wt/wt
HL 43	M	41	diazepam	320	wt/wt
HL 44	F	49	—	1750	n.d.*
HL 45	M	46	pentobarbital	2260	wt/wt
HL 46	M	—	—	1070	wt/wt
HL 48	M	61	—	2420	wt/wt
HL 49	M	49	—	980	wt/wt
HL 50	M	27	—	410	wt/wt
HL 51	F	69	—	980	wt/wt
HL 52	M	23	clindamycin, benzylpenicillin, gentamycin	310	wt/wt
HL 53	M	22	marijuana smoker	1620	wt/wt
HL 54	F	28	—	1850	n.d.*
HL 55	M	28	no drug intake, no alkohol	1830	wt/wt
HL 109	F	56	felodipine	970	wt/wt
HL 110	F	40	smoker, paroxetin	2380	wt/**mut**
HL 112	F	66	no drug intake, non smoker	770	wt/wt
HL 113	F	62	—	2700	wt/wt
HL 115	F	34	non smoker	1090	wt/wt
HL 116	F	75	thyroxine, isorbide dinitrate, metoprolol, acetylsalicyclic acid	2360	wt/wt
HL 117	M	61	smoker	1380	wt/wt
HL 118	M	56	non smoker	2610	wt/**mut**
HL 119	M	70	—	1480	wt/wt
HL 120	F	78	metoprolol, diazepam	1130	wt/wt
HL 121	M	61	non smoker	1330	wt/wt
HL 122	M	63	—	1230	wt/wt
HL 123	M	67	—	950	wt/wt
HL 124	M	62	—	2290	wt/wt
HL 126	M	67	—	1800	wt/wt
HL 127	F	60	metoprolol, non smoker	1730	wt/wt
HL 128	F	68	hydrochlorothiazide, non smoker	1630	wt/wt
HL 129	F	61	citalopram, diazepam	1960	wt/wt
				mean $\pm$ s.d.=	
22 Females				2460 $\pm$ 2250	
24 Males				2005 $\pm$ 1870	
46 Total				2220 $\pm$ 2050	

* not determined, [a]homozygous wildetype, [b]heterozygous for the V-allele, [c]homozygous for the V-allele, [d]average of duplicate determination.

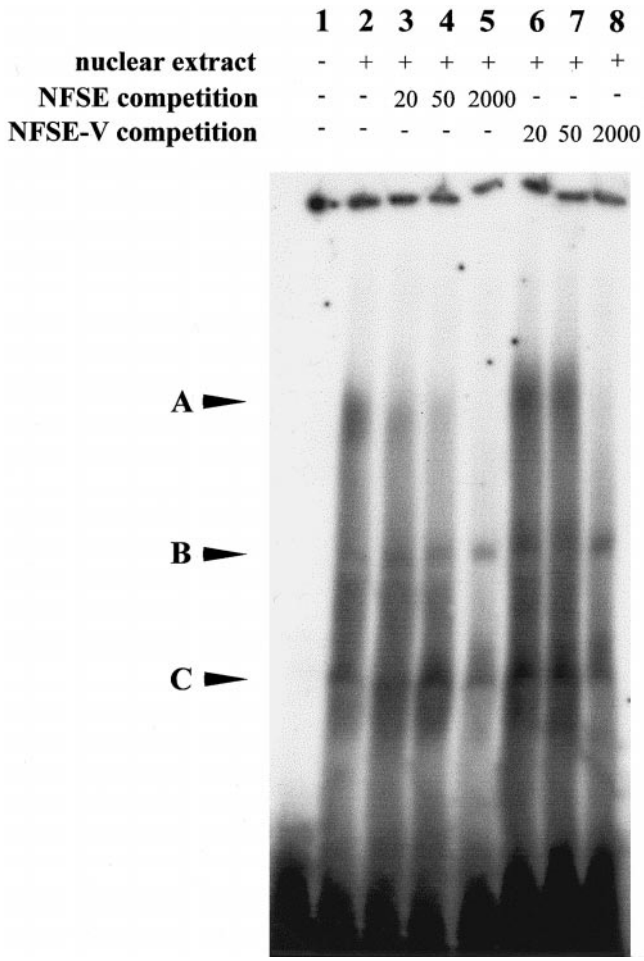

FIG. 1. Gel-shift assay for the NFSE of CYP3A4 gene. 15 fmol of double stranded ^{32}P-labeled NFSE probe together with 20 μg nuclear extracts was used in each binding reaction. Free probe is shown in lane 1 and the NFSE-protein complex in lane 2. In competition experiments, 20, 50, and 2000 fold molar excess of unlabeled NFSE (lanes 3, 4, and 5) and 20, 50, and 2000 fold molar excess of unlabeled NFSE-V (lanes 6, 7, and 8) were used, respectively. The low mobility complex (A) and the two high mobility complexes (B and C) are indicated with arrows.

In order to evaluate any importance of the −290 A→G mutation for specific binding of nuclear proteins, gel mobility shift experiments were carried out using nuclear extracts from human livers and oligonucleotides corresponding to wild type and mutated forms of NFSE. Control experiments using a TATA-containing oligonucleotide revealed specific binding of proteins in these particular nuclear extracts fully competed with 20-fold molar excess of unlabeled oligo (data not shown). As shown in Fig. 1, three different protein complexes binding to NFSE were seen. Two bands with high mobility were reproducibly seen independently of ionic strength. However, since these bands were not competed using 2000-fold excess of unlabeled oligonucleotide they represent non specific protein binding. A low mobility protein complex was also seen binding to wildtype NFSE, but it could not be fully competed using as much as a 50-fold molar excess of unlabeled NFSE oligo, whereas a 2000-fold excess resulted in full competition. The low mobility gel shift required low ionic strength (50 mM KCl) in the binding reaction and as much as 20 μg of nuclear extracts was needed. Despite using as much as 100 000 cpm per 15 fmol probe and lane, the complex was only visualised after 3-4 days exposure. Taken together, these findings indicate that the low mobility complex is of very low abundance and is bound with low affinity. The binding affinity of nuclear proteins to the mutated NFSE oligo (NFSE-V) was even weaker as shown by reduced competition at 20 and 50-fold molar excess of unlabeled NFSE-V compared to NFSE, but a 2000-fold excess of unlabeled NFSE-V oligo provided full competition.

DISCUSSION

The present study shows that the promoter of the CYP3A4 gene is highly conserved. Sequence analysis of 78 different alleles revealed no other single mutations or sequence variations found between +10 and −490 except for the previously reported base pair mutation in the CYP3A4 specific element NFSE. The functional properties of this variant allele was evaluated and the hepatic *in vitro* CYP3A4 activity data from individuals heterozygous for the V-allele suggests that the mutation does not significantly affect the catalytic activity of the enzyme as measured in isolated liver microsomes. A large (31-fold) interindividual variation in the CYP3A4 activity in the material of 46 human liver samples was seen which is in agreement with previous studies (1, 3, 19). The *CYP3A4* gene expression is controlled by transciptional factors such as the pregnenolone X receptor (hPXR), the steroid and xenobiotic receptor (SXR) and the glucocorticoid receptor (8, 9, 20). The ligands for these receptors include glucocorticoids, drugs like nifedipine, phenytoin and rifampicin as well as xenobiotics like PCBs. A major factor for interindividual variation in the gene expression might thus be caused by the occurrence of different levels of such activated ligands and/or receptors within the different individuals. It is therefore likely that these exogenous and endogenous factors influence the *CYP3A4* gene expression to a much higher extent than the genetic polymorphism here studied of which effects, if any, are overshadowed by these factors. In this context it should be pointed out that our material includes liver samples from donors known to have been taken drugs which induce CYP3A4 such as glucocorticoids and barbiturates (17, 18).

The previously suggested functional importance of the *CYP3A4-V* polymorphism did not receive support from the results of our gel shift experiments using an oligonucleotide corresponding to wildtype NFSE element. The only protein complex detectable that could bind to the NFSE element with some specificity did so with low af-

finity characteristics as it was undetectable in the presence of 120 mM KCl. Furthermore, the low mobility complex could not be effectively competed by a 20 or 50-fold molar excess, as is expected for a binding of high affinity and specificity. These results together with the finding of a very low abundance of the protein complex detected suggest that the NFSE element may not be of high importance for CYP3A4 expression. These data are in agreement with those originally presented by Hashimoto et al. (7) who also performed the gel-shift assay under low ionic strength conditions. The affinity of the complex they detected however, is not possible to estimate since they only showed results from competition experiments using as much as 2000-fold excess of unlabeled oligo. Thus, even though nuclear protein appear to have an even lower affinity for the mutated oligonucleotide than for the wildtype variant, as shown by competition experiments using both the wildtype and mutated oligo (Fig. 1), the NFSE is unlikely to be of high significance for CYP3A4 expression.

In recent epidemiological studies, associations with higher clinical stage and grade of prostate cancer and treatment related leukaemia are made based on the speculation that the *CYP3A4-V*-allele causes a decrease in CYP3A4 expression and activity. Based on the results that 21% of patients suffering of *de novo* leukaemia's with MLL gene translocations but none out of 22 epipodophyllotoxin treatment related leukaemia's with MLL gene translocations carried the CYP3A4-V allele, Felix et al. (6) concluded that subjects carrying the wildtype allele are at increased risk for this cancer and that epipodophyllotoxin metabolism by CYP3A4 should contribute to secondary cancer risk. In their assumption, the expression of the CYP3A4 in carriers of the V-allele should have been decreased, which does not get support from our study. A similar relationship was proposed by Rebbeck et al. (4) who found a higher incidence of the variant *CYP3A4* allele in men with prostate tumours with higher clinical stage and grade and assumed the CYP3A4-dependent metabolic pathway of testosterone in prostate carcinogenesis to be affected by the *CYP3A4-V* polymorphism, also this a finding is without support from our study. Indeed, it is striking that the V-allele frequencies determined by Rebbeck et al. (4) are so high (9.6%) and not compatible with those here obtained only using sequence analysis for its determination. This could indicate methodological difficulties in the conformation-sensitive gel electrophoresis method used for detection.

In conclusion, our data indicate that the polymorphism in the CYP3A4 NFSE-element does not constitute a factor of importance to explain the high interindividual variability in CYP3A4 expression. This variation is apparently caused by other factors, most likely an interindividual difference in the levels of transcriptional activators of importance for the CYP3A4 gene expression. This suggests no linkage between the distribution of the *CYP3A4-V* allele with the risk for prostate cancer or incidence of treatment related leukaemia, in contrast to previous conclusions (4, 6).

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