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cDNA AND DEDUCED AMINO ACID SEQUENCE OF A DWARF GOAT LIVER CYTOCHROME P450 FRAGMENT BELONGING TO THE CYP2C GENE SUBFAMILY+

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From a liver cDNA library derived from a phenobarbital treated dwarf goat a 1176 bp cDNA-fragment, coding for 285 amino acids, has been isolated. Northern blot analysis reveals detection of a 2 kb mRNA which is inducible by phenobarbital, triacetyloleandomycin and to an lesser extent by ß-naphthoflavone. Analysis of the deduced amino acid sequence shows a 72.5% and a 71.1% homology with rabbit CYP2C3 and human CYP2C17, respectively. The nucleotide sequence reveals homologies of 79.8% and 78.7%, respectively. Comparison of the amino acid sequences between CYP2C forms of various species reveals a high homology around the Cys_{436}, the so-called C-terminal cysteine containing peptide, which is assigned to be the heme-binding region.

Cytochrome P450 (P450), the major catalytic compound of the mixed-function oxidase system, plays a crucial role in the metabolism of many endogenous and exogenous compounds. On the basis of amino acid sequences P450s are classified into gene families (1). Families 1 to 4 cover the major drug-metabolizing P450s. These enzymes appear to be inducible by more or less selective xenobiotics. Inducibility, in combination with selective metabolic activities, has provided a basis for classification and characterisation. However, there are marked qualitative and quantitative differences in the presence, activity and inducibility of P450 enzymes for various species, such as rat, rabbit and man (2,3). Recently this has also been reported for the West African dwarf goat (*Capra hircus aegagrus*) (4). For instance, CYP2E may be of constitutive

+This sequence is submitted to Heidelberg database and received the accession number X76502.

Abbreviations: PB, sodium phenobarbital; BNF, ß-naphthoflavone; TAO, triacetyloleandomycin.

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importance in dwarf goats since high portal blood concentrations of ketone bodies, shown to induce CYP2E in rats (5), are inherent to ruminant physiology. This view seems to be supported by Northern blots of RNA from untreated goats, in which a strong hybridization signal is seen using a human CYP2E1 cDNA as hybridization probe (Horbach & van 't Klooster, unpublished results). As part of a long-term effort to elucidate the structural and functional properties of the dwarf goat cytochrome P450 enzymes we have initiated efforts to clone P450 cDNAs.
We report here the nucleotide and deduced amino acid sequence of a cDNA-fragment that is found after screening a dwarf goat liver cDNA library with human CYP2E1. Sequence analysis of this cDNA revealed that it should be assigned to the CYP2C subfamily rather than the CYP2E subfamily.

Materials and Methods

Materials
Sodium phenobarbital was obtained from Brocacef (Maarssen, The Netherlands). ß-Naphthoflavone was obtained from Sigma (St.Louis, MO, USA). Triacetyloleandomycin was a kind gift from Pfizer (Rotterdam, The Netherlands).The cDNA probe pUC-h2E1 (human P4502E1) was a kind gift from Dr. F.J. Gonzales (Bethesda, MD, USA).

Animal treatment
Three months prior to the experiment, 3 female (11-15 kg) dwarf goats, 8-10 months of age, were housed under controlled conditions. Animals were fed a commercially available pelleted feed concentrate and had free acces to hay and water. PB (dose: 50 mg/kg.day) and TAO (100 mg/kg.day) were administered orally, by means of 2 daily administrations. BNF (20 mg/kg), dissolved in arachid oil, was administered by daily subcutaneous injections at four different and changing locations in the flanks and the neck region. Treatment with inducers was performed for 6 consecutive days, including the day of termination. The livers were removed within 10 minutes after sacrificing, perfused with icecold saline and frozen in liquid nitrogen.
The Ethics Committee for animal experimentals of the Veterinary Faculty has approved the use of the animals in the present experiment.

Isolation of RNA and construction of a cDNA library
Liver material from a PB-induced female dwarf goat was used for the construction of a cDNA library in the shuttle plasmid pSV-SPORT1 (BRL, Life Technologies, Gaithersburg, MD)(6). Total RNA was isolated from saline-perfused, liquid-N_2 frozen dwarf goats liver using the LiCl/urea procedure of Auffray (7) as modified by Clemens (8), with an additional phenolic extraction step. Purified poly(A)-mRNA was obtained using oligo(dT)-cellulose column chromatography (BRL)(9).
Double stranded cDNA was synthesized using oligo(dT)/NotI primed poly(A)-mRNA and Moloney Murine Leukemia Virus reverse transcriptase followed by RNaseH and DNA polymeraseI. Blunt ended DNA was prepared using T4 DNA polymerase, ligated with EcoRI adaptors (Pharmacia, Milwaukee, WI), digested with NotI and ligated with NotI/EcoRI-digested pSV-SPORT1.

Isolation and sequencing of goat cDNA clones
On Hybond N^+-membranes (Amersham) replica's of cDNA containing bacteria (DH5α) were made and hybridized with random primed [α^{32}P]dCTP-labelled human CYP2E1 cDNA.

Hybridization was carried out overnight at 65°C in the presence of 7% w/v SDS in 0.5 M phosphate buffer (Ph 7.2), using random-primed [α^{32}P]DCTP-labelled probes. Following hybridization filters were washed at 65°C in 2*SSC (1*SSC is 150mM NaCl in 15mM sodium citrate, Ph 7.0), 0.1% SDS (2 x 30 min), followed by 1*SSC, 0.1% SDS (2 x 30 min). Autoradiography was performed at -80°C using Hyper-film (Amersham, Buckinghamshire, UK). DNA from positive clones was isolated using the Ammoniumacetate-method (10). The largest positive clone was sequenced using the dideoxy chain terminator method (11), with use of the a T7 sequencing kit (Pharmacia, Milwaukee, WI). Sequence was displayed on a 5% polyacrylamide, 50% urea gel without a salt gradient.

RNA isolation and Northern blots
RNA was isolated as descibed above. Equal amounts of total RNA (15 µg per lane) were size-fractionated by electrophoresis in a 1.5% agarose gel containing 2.2 M formaldehyde followed by transfer to a Hybond-N^+ membrane (Amersham, Buckinghamshire, UK). Hybridization conditions were as described above with to extra washing steps at 65°C with 0.3*SSC,0.1%SDS (1 x 30 min) and 0.1*SSC, 0.1%SDS (1 x 30 min).

Results and Discussion

Using mRNA isolated from phenobarbital-treated dwarf goat liver, cDNA was synthesized and incorporated between the EcoRI and NotI site of pSV-SPORT1 and cloned. A cDNA library of 8000 colonies was obtained and screened with human CYP2E1 cDNA. The positive clones contained cDNAs ranging from 400-1200 bp. The largest positive clone was selected for sequence analysis. Translation of the identified base-pair sequence into amino acids revealed an open reading frame of 285 amino acids (Figure 1).

Comparison of this deduced amino acid sequence, with the SwissProt data-library, suprisingly, showed a higher homology with members of the CYP2C subfamily (ranging from 66.1% to 72.5%) than with the CYP2E subfamily (maximal 58.7%) (Table I). The highest amino acid homology was found with rabbit CYP2C3 (12) and human CYP2C17 (13), 72.5% and 71.1%, respectively (Figure 1). Comparison of the nucleotide sequence of the goat cDNA with these CYP2C forms revealed a homology of 79.8% and 78.7% for rabbit CYP2C3 and human CYP2C17, respectively (Figure 1).

A large region, around the Cys_{436} in the human CYP2C17 sequence, is conserved remarkably between the species. A more extensive comparison of the sequence of this C-terminal cysteine containing peptide, which is assigned to be the most suitable place for the heme-binding site (14), is given in figure 2. Only 6 amino acids are different when all given CYP2C sequences are compared, position +1 of the cysteine can be changed but this is always into another hydrophobic residue. The homology is more striking when goat CYP2C is compared with either rabbit CYP2C3 or human CYP2C17, only 2 differences are found. When this highly conserved region is compared with other members of the CYP2 family, for example CYP2E1 or rabbit CYP2B4, more or less the same conserved bases are found. Comparison with human CYP3A4 shows that there is no conservation of this region between different families.

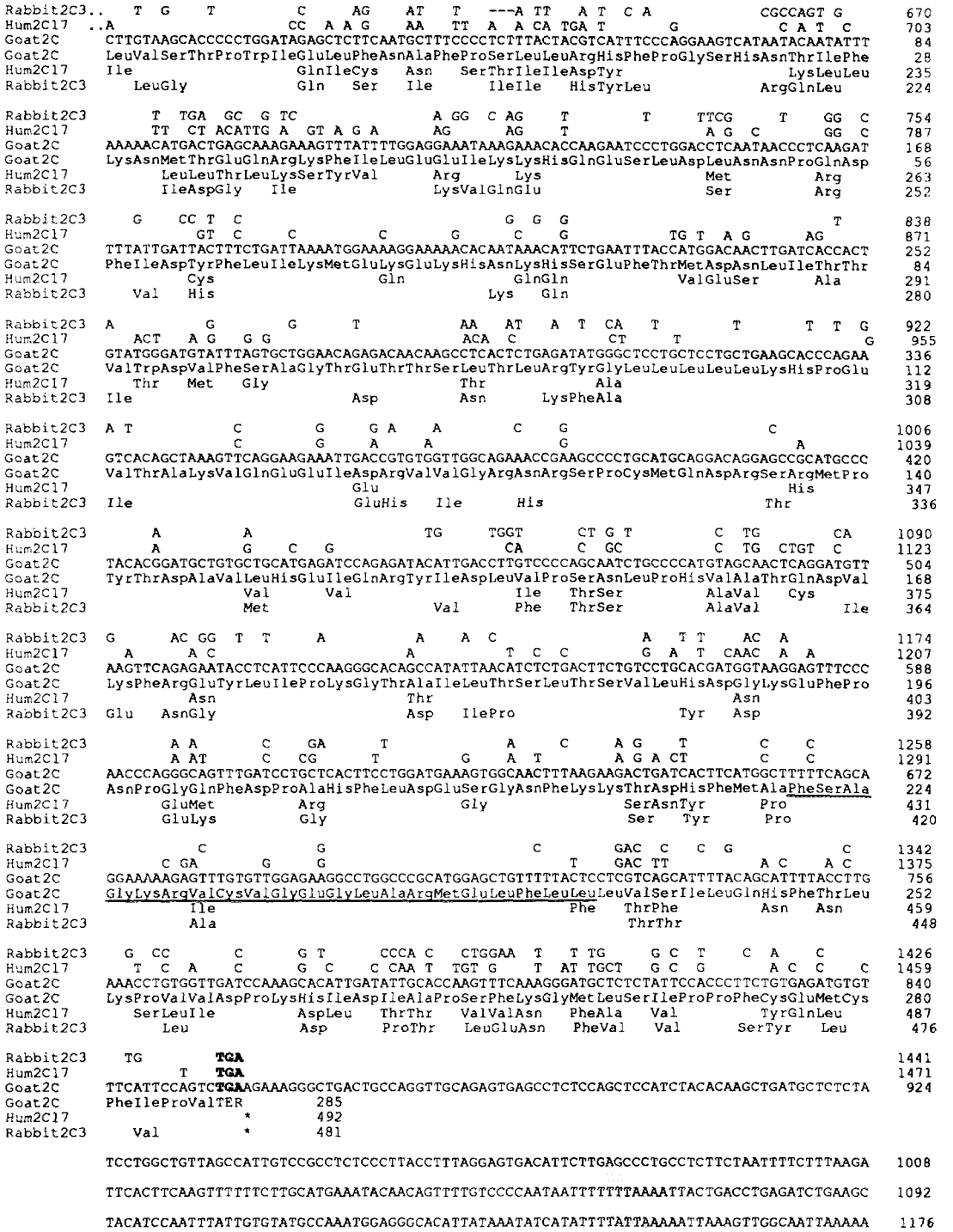

Figure 1. Nucleotide and deduced amino acid sequence of goatCYP2C (in the middle) and comparison with humanCYP2C17 (13) and rabbitCYP2C3 (12). Only the differences form goatCYP2C are indicated in these sequences. The nucleotide sequence of humanCYP2C17 and rabbitCYP2C3 are terminated behind the termination codon. The C-terminal cysteine containing peptide is underlined.

Table I: Sequence homology between the isolated fragment and CYP forms of different species

Species	Gene number	Trivial name	% Homology		Reference
			amino acid	nucleic acid	
Rabbit	2C1	PBc1	66.1		12
	2C3	PBc3	72.5	79.8	12
	2C4	PBc4	66.2		15
Rat	2C6	P450k	66.9		16
	2C11	P450h	68.3		17
Human	2C8	IIC2	66.5		18
	2C9	IIC1	66.1		19
	2C17	254c	71.1	78.7	13
	2C18	29c	69.4		13
	2C19	11a	68.7		13
	2E1	P450j	58.7	65.8	20

Northern blot analysis of PB, TAO and BNF treated dwarf goats with the isolated cDNA fragment revealed hybridization with a mRNA of 2 (Figure 3). This mRNA is inducible by PB, TAO and to a lesser extent BNF. PB induction of CYP2C mRNA is also described in rabbits by Leigthon et al (12). TAO and BNF, on the other hand, are no CYP2C inducers in mammalian species studied so far. However, it should be noted that our findings were obtained in individual goats. Intra-species differences in CYP2C expression might also be responsible for these observations.

In conclusion, we have isolated an P450 cDNA-fragment from the CYP2C subfamily that is closely related to CYP2C3 from rabbit and humanCYP2C17. Homology to another CYP2C members is ranging from 66.1% to 72.5% and indicates that this goat form should indeed be assigned to the CYP2C subfamily.

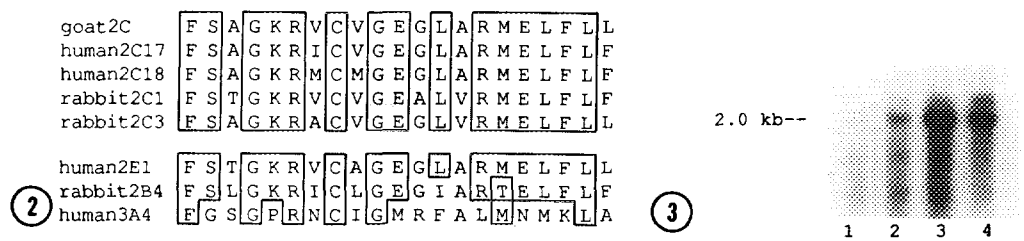

Figure 2. Comparison of deduced amino acid sequences in the heme binding cysteine containing peptide. Important similarities between the sequences are boxed.

Figure 3. Northern blot analysis of induced goat liver with goat CYP2C cDNA probe.
15 µg of liver microsomal RNA of treated and untreated goats was hybridized with the isolated goat CYP2C fragment (lane 1: control; lane 2: BNF-induced; lane 3: PB-induced; lane 4: TAO-induced).

Acknowledgments

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