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Cloning and Initial Characterization of the Human *DPYD* Gene Promoter

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Dihydropyrimidine dehydrogenase (DPD) is the rate-limiting enzyme in the degradation of pyrimidine bases and pyrimidine-based antimetabolites. Reduced DPD activity is associated with toxicity to 5-fluorouracil (5FU) therapy in cancer patients and with neurological abnormalities in paediatric patients. Although variant *DPYD* alleles have been identified in DPD-deficient patients, they do not adequately explain polymorphic DPD activity or associated clinical phenotypes *in vivo*. DPD may be transcriptionally regulated as mRNA levels correlate with activity and are differentially regulated in human tissues. A 1.85 kb 5′ flanking region of the human *DPYD* gene was cloned and has transcriptional activity in cultured cells. Analysis of this 5′ flanking region in rhesus and cynomolgus monkeys demonstrated conservation (>96%) between humans and primates. Putative binding sites for ubiquitous and cell-specific factors were identified. A polymorphism that disrupts a putative γ-interferon response element was identified in a cancer patient with reduced DPD activity and severe 5FU toxicity. Further insight into regulation of DPD expression may identify new avenues for the treatment of clinical problems associated with DPD deficiency.

Key Words: **dihydropyrimidine dehydrogenase; promoter; 5-fluorouracil; toxicity; transcription; human; rhesus; cynomolgus; polymorphism.**

Dihydropyrimidine dehydrogenase (DPD) is the first and rate limiting enzyme in the degradation of the pyrimidine bases, uracil and thymine (1). This catabolic pathway is the sole endogenous source of β-alanine in mammals. In addition, over 80% of an administered dose of the antimetabolite 5-fluorouracil (5FU) is inactivated via this pathway (2). 5FU is commonly used in the treatment of colorectal, breast and head/neck cancers (3). However, response rates to 5FU remain relatively low, probably reflecting the variable metabolism of the drug.

DPD demonstrates considerable variation (8- to 21-fold) in healthy and cancer populations and 3–5% of individuals have reduced DPD activity, associated with severe, sometimes life-threatening 5FU toxicity in cancer patients (4–6). Complete DPD deficiency is also associated with the inherited metabolic disorder thymine-uraciluria which is characterised by neurological problems in paediatric patients (7).

DPD is encoded by the *DPYD* gene on chromosome 1p22 and 17 *DPYD* mutations have been reported to date (8, 9). These variant alleles were identified in DPD-deficient paediatric patients with neurological abnormalities or in cancer patients with reduced DPD activity and 5FU intolerance (10–12). However, these alleles do not adequately explain either polymorphic DPD activity *in vivo* or the majority (>85%) of cases of reduced DPD activity in cancer patients with 5FU toxicity (9).

There is limited information on the factors that regulate DPD activity. Regulation of DPD activity by nutritional factors or enzyme substrate (5FU) has been previously described *in vivo* (13, 14). Tissue-specific DPD expression is apparent with highest activity in liver and peripheral blood mononuclear cells (PBMC) and reduced activity in other major organs (15). DPD mRNA levels are reduced in colon compared to liver consistent with pre-translational control of DPD expression (16). In addition, a direct relationship between mRNA levels and DPD activity was observed in normal and tumour tissues (16, 17). These data indicate that DPD phenotype is controlled, at least in part, by a transcriptional or post-transcriptional mechanism in normal and neoplastic human tissues. In this study we report the cloning and initial functional characterisation of the 5′ flanking region of the human *DPYD*

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TABLE 1

Primers Used for RAGE, PCR and Sequencing Analysis of the Human and Monkey *DPYD* Genes

pT7	5′ TAATACGACTCACTATAGGG 3′
pT7nest	5′ CTCGAGGTCGACGGTATC 3′
pE1F	5′ GCTGTCACTTGGCTCTCT 3′
p111X	5′ CTCGATGTCCGCCGAGTCCTTGCT 3′
pDPYD1	5′ TGTCCGCCGAGTCCTTAC 3′
pDPYD2	5′ GCCATGGCAGTGCCTACA 3′
pDPYD2B	5′ CCACCTCGCTGGGACCTACTG 3′
pDPYD3	5′ AGTCTCCTTCAACAATATGAGGCTT 3′
pDPYD4	5′ TTCATCCCTCTTGTTTCCCAGGT 3′
pDPYD4B	5′ ACCTGGGAAACAAGAGGGATGAA 3′
pDPYD5	5′ CTGTGTGATCTTGGGGAAGTCAC 3′
pDPYD6	5′ GCTGTATCTATAATTCCAGAC 3′
pDPYD7	5′ TGAGTGTCTTCGTCTACTAATGGTTTC 3′
pDPYD8	5′ TGATTTCTTTTCTCATTATAGGTAAAT 3′
pDPYD9	5′ ATTTACCTATAATGAGAAAAGAAATCA 3′
pDPYD10	5′ ATGGGCACTTCTGGGATCTGACCAA 3′
pDPYD11	5′ AAACATATCCCAGTGAACCCAACTC 3′
pDPYD12	5′ CCCTGACTTGCTCCACCCCCTTGGC 3′
pDPYD13	5′ AGAAGAGGAAACTGGCTCAGAAA 3′
pDPYD14	5′ AAATCATTGCTAGTAATCTCTGT 3′

gene. This region was also analysed in cynomolgus and rhesus monkeys to provide insight into conserved regions of putative functional importance.

MATERIALS AND METHODS

Samples. Following informed consent, whole blood was collected from 37 cancer patients (18 men, 19 women) with reduced (20–146 pmol/min/mg protein; mean 95 pmol/min/mg protein; $n = 23$) or normal (173–482 pmol/min/mg protein; mean 250 pmol/min/mg protein; $n = 14$) PBMC DPD activity (18, 19). Whole blood was also collected from unrelated blood donors from Aberdeen, UK. Genomic DNA was extracted from human whole blood and from the livers of two rhesus and two cynomolgus monkeys using the Nucleon II extraction method (Scotlab, Coatbridge, UK).

Rapid amplification of DNA ends (RAGE). The 5′ flanking sequence of the human *DPYD* gene was determined using rapid amplification of genomic DNA ends (RAGE), essentially as previously described (20). Human genomic DNA (5 μg) and SK^+-bluescript plasmid DNA (5 μg) were each digested separately with *Eco*RI, *Bam*HI, *Hin*dIII, *Xho*I, *Xba*I or *Pst*I and purified as described previously (20). Ligation of the human DNA to dephosphorylated SK^+-bluescript, created six libraries of *Eco*RI, *Bam*HI, *Hin*dIII, *Xho*I, *Xba*I or *Pst*I digested human genomic DNA ligated to the multiple cloning site of SK^+-bluescript.

Six 50 μl PCR reactions each containing 2 μl of a human/SK^+-bluescript library in 1× buffer (Promega, WI) with 2 mM $MgCl_2$, 1 mM dNTPs, 25 pmol of pT7, a plasmid-specific primer (Table 1) and 25 pmol of pDPYD1, a *DPYD*-specific primer (Table 1) were denatured at 94°C for 3 min before the addition of 2.5 U *Taq* polymerase. The DNA was amplified using 31 cycles of 94°C 40 s, 52°C 1 min and 72°C 3 min, followed by a 15 min final extension step at 72°C. A second round of PCR was performed using 0.1% of the first PCR reaction as template. The same conditions were used except 1 mM $MgCl_2$, 1.25 U *Taq* polymerase and an annealing temperature of 55°C were used in the PCR with two nested primers, pT7nest and pDPYD2 (Table 1).

A 0.6 kb fragment was successfully amplified from the *Eco*RI library (Fig. 1). This fragment was purified using Centricon microconcentrators (Amicon, Stonehouse, UK) and both strands were sequenced on an automated ABI377 DNA sequencer using either pT7nest or pDPYD2 and a rhodamine-based dideoxy-terminator mix (Applied Biosystems, CA).

A second round of RAGE used primers pDPYD3 and pDPYD4 (Table 1), which were designed at the 5′ end of the novel *DPYD* 5′ flanking sequence obtained using pDPYD1 and pDPYD2. Amplification with pDPYD3 and pT7 was followed by nested PCR with pT7nest and pDPYD4 as described above. A 1.4 kb fragment was generated from the *Hin*dIII library (Fig. 1) and both strands of this fragment were sequenced as described above. Smaller fragments amplified from the other libraries confirmed the sequence obtained from the *Eco*RI and *Hin*dIII libraries in the two rounds of RAGE analysis.

Analysis in humans and monkeys. The 1.85 kb human *DPYD* 5′ flanking sequence was confirmed using *DPYD*-specific primers to amplify 7 overlapping fragments by PCR in genomic DNA from 3 unrelated blood donors (Table 1 and Fig. 1). This PCR analysis was also used to screen 37 cancer patients for *DPYD* polymorphisms and

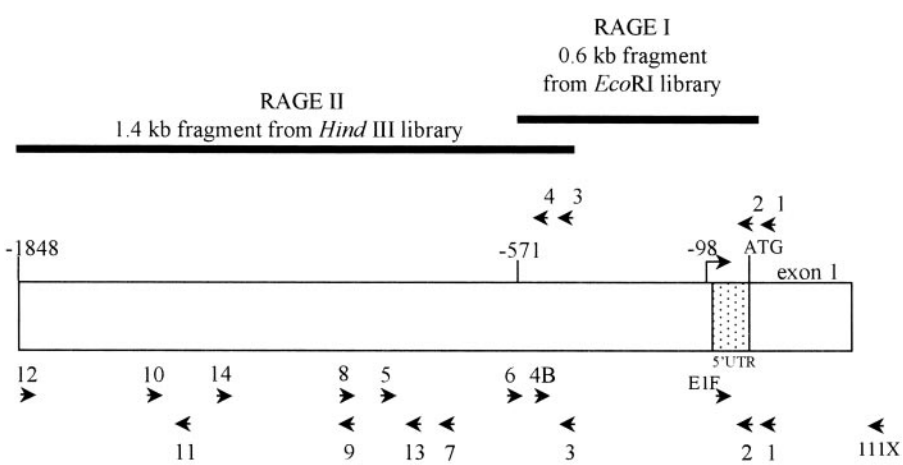


FIG. 1. Schematic diagram of RAGE and PCR analysis used to obtain the 5′ flanking sequence of the human *DPYD* gene. RAGE analysis (illustrated above the gene) provided 1767 bp of novel sequence (GenBank Accession No. AF214571) and 86 bp of previously published exon 1 *DPYD* sequence (Accession No. HSO9178). This sequence was confirmed in genomic DNA from 3 individuals using 8 primer sets, pE1F/111X, pDPYD1/6, pDPYD2/4B, pDPYD3/5, pDPYD7/8, pDPYD9/10, pDPYD11/12 and pDPYD13/14 (illustrated below the gene).

to obtain the 5′ flanking sequences of the rhesus and cynomolgus monkey *DPYD* genes.

For primer sets pE1F/p111X, pDPYD3/5, pDPYD7/8, pDPYD9/10 and pDPYD11/12 and pDPYD13/14 (Table 1), 1 μl genomic DNA was amplified in 1× buffer (Promega, WI) in the presence of 1–2.5 mM $MgCl_2$, 1 mM dNTPs, 1 U *Taq* polymerase and 100 ng of each primer in a total volume of 50 μl. For primers pDPYD13/14, 10% DMSO was included. DNA was denatured for 3 min at 94°C before amplification and a final 5–10 min extension step at 72°C was included. DNA was amplified using 31–35 cycles of 94°C for 40 s, 50–60°C for 30–60 s and 72°C for 30–90 s.

The region immediately upstream of the translation start site was amplified using nested PCR. A 50 μl PCR reaction containing, 2 μl of genomic DNA in 1× buffer (Promega, WI), 2.5 mM $MgCl_2$, 1 mM dNTPs, 1 M betaine and 25 pmol each of pDPYD1 and pDPYD6 (Table 1), was denatured at 94°C for 3 min, before the addition of 2.5 U *Taq* polymerase. The DNA was amplified using 35 cycles of 94°C for 40 s, 52°C for 60 s and 72°C for 90 s, followed by a 15 min final extension step at 72°C. Nested PCR was performed using 0.1% of the first PCR reaction as template. The same conditions were used except 1.25 U *Taq* polymerase and an annealing temperature of 54°C were used in the PCR with two nested primers, pDPYD2 and pDPYD4B (Table 1). Both strands of each PCR product were sequenced as described above.

Cloning. 5′ flanking regions of the *DPYD* gene were cloned into pCR2.1 vector (Invitrogen, Groningen, The Netherlands) following amplification by PCR. A region from −1848 to −183 (numbered from the translation start site at +1) was amplified from human genomic DNA in 1× buffer (Promega, WI) in the presence of 1.5 mM $MgCl_2$, 1 mM dNTPs, 1 M betaine, and 100 ng each of pDPYD12 and pDPYD2B (Table 1). DNA was amplified for 31 cycles of 94°C for 40 s, 55°C for 1 min and 72°C for 3 min, following denaturation at 94°C for 3 min and addition of 1.25 U *Taq* polymerase. A final extension step at 72°C for 10 min was included. A fragment from −519 to +5 was amplified by nested PCR using pDPYD1/6 and pDPYD2/4B as described above.

The PCR fragments were cloned into pCR2.1 vector using T4 DNA ligase at 16°C overnight. The orientation of the inserts was determined by restriction enzyme mapping. A 2 kb *Bsp*LU11I fragment was isolated from pCRDPYD −1848/−183 and was ligated to a 3.8 kb *Bsp*LU11I fragment isolated from pCRDPYD −519/+5, to create a construct containing a 1.85 kb *DPYD* 5′ flanking fragment (−1848 to +5). A *Sac*I/*Xho*I *DPYD* fragment (−1848 to −21) was subcloned into the pGL3-Basic luciferase reporter vector (Promega, WI) [pGLB1848].

Cell culture. Hs766T cells (ATCC HTB-134; human pancreatic carcinoma metastatic to lymph node) and HepG2 cells (ECACC 85011430, human hepatocyte carcinoma) were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium with 4.5 g/L glucose (DMEM) containing 10% fetal bovine serum and 2 mM glutamine. Medium was renewed twice weekly and cells were subcultured at subconfluence. Endogenous DPD activity was measured in Hs766T and HepG2 cells using a ^{14}C-5FU HPLC method as previously described (18).

Transfections. Plasmid DNA (2 μg) was incubated with 24 nmol liposomes (1,2-dioleoyl-*sn*-glycero-3-phospho-ethanolamine (Fluka, Buckinghamshire, UK): dimethyldioctadecyl ammonium bromide (Sigma, Poole, UK); 1:0.5) in DMEM for 30 min at room temperature. Cells, subcultured into 6-well plates 24 h prior to transfection, were then incubated with the DNA:liposome mix for 5 h at 5% CO_2 and 37°C. Fetal calf serum was added to the cells at a final concentration of 10% and the cells were cultured for a further 48 h. The cells were washed and harvested by scraping in 1 ml cold phosphate buffered saline, pH 7.4. Following centrifugation at 6000 rpm for 1 min, cells were resuspended in 50 μl 100 mM KH_2PO_4, pH 7.8. The cells were lysed by freeze-thawing and the cell debris was removed by centrifugation at 14000 rpm for 30 s. Luciferase activity (relative light units; RLU) in cell cytosols was measured in buffer A (30 mM glycylglycine, 15 mM $MgSO_4$, 2 mM ATP, 450 μM coenzyme A, 2.6 mM Triton X-100) in the presence of D-luciferin (40 μM) on a Berthold Luma LB9501 luminometer. The amount of protein in each cytosol was measured using the Bradford assay (21) and luciferase activity was corrected for protein.

Restriction fragment length polymorphism. PCR-RFLP was used to analyse a mutation at −1590 in the *DPYD* gene. A 429 bp fragment, amplified by PCR using primers pDPYD11 and pDPYD12 as outlined above, was digested with *Mse*I and separated on a 2% agarose gel to yield fragments of 259 and 170 bp. The T-1590C mutation destroys a *Mse*I site within this fragment, allowing RFLP analysis of the digested products.

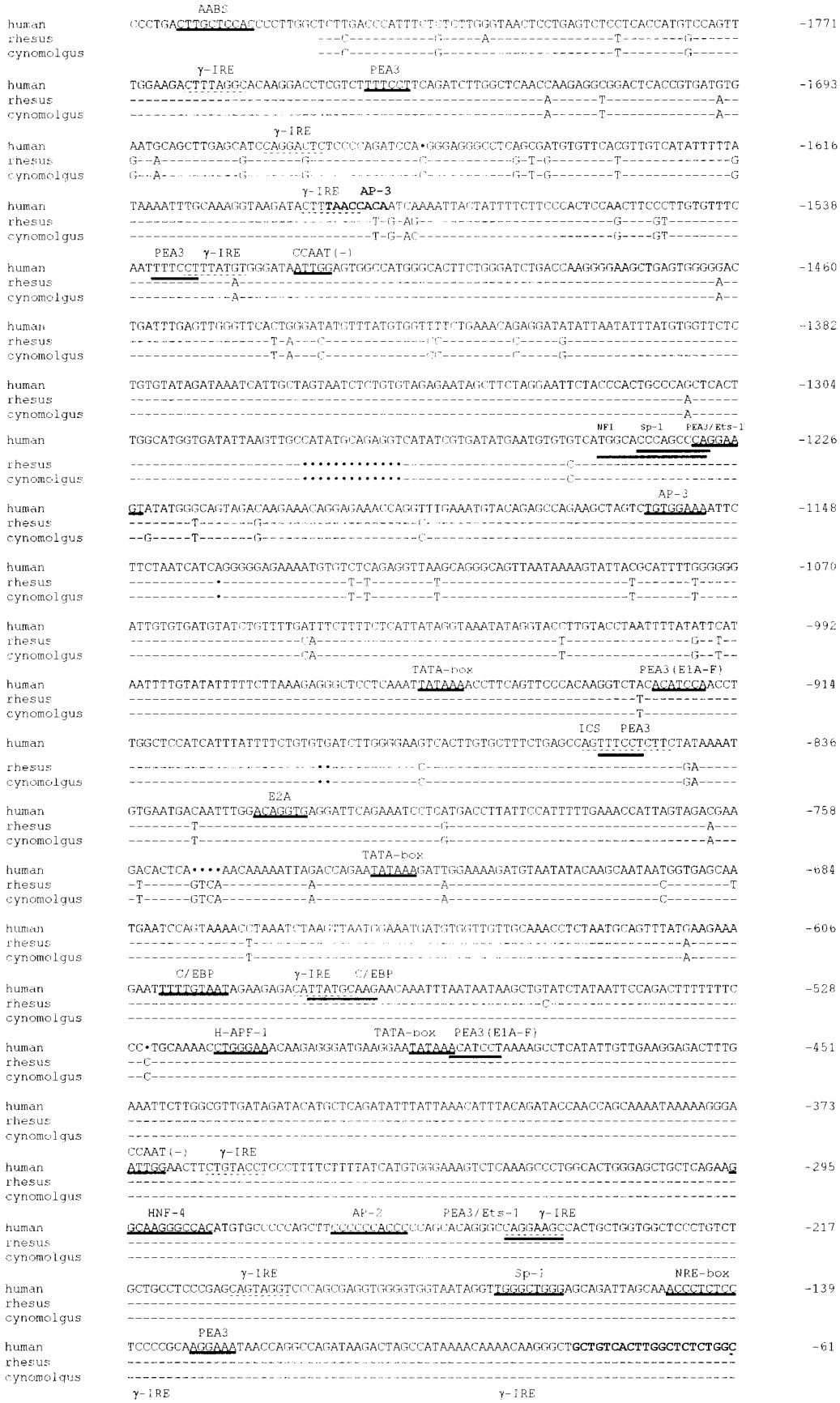
Computer analysis. The 1.85 kb 5′ flanking region of the *DPYD* gene was compared to sequences in the GenBank/EMBL and EST databases using GCG at www.hgmp.mrc.ac.uk. Putative TATA boxes, CCAAT boxes and transcription factor binding sites were identified using FINDPATTERNS in GCG to search the transcription factor database. The human *DPYD* sequence was aligned with the monkey sequences using ALIEN (http://www.hgmp.mrc.ac.uk).

RESULTS

Rapid amplification of genomic DNA ends (RAGE) was used to obtain 1.85 kb of sequence upstream of the translation start site in the human *DPYD* gene (GenBank Accession No. AF214571; Figs. 1 and 2). This region contained 1767 bp of novel sequence upstream of the previously published 5′ terminal base of the human *DPYD* cDNA and 86 bp of previously published exon 1 sequence. The wild type sequence was confirmed in 3 unrelated individuals by sequencing 7 overlapping PCR fragments amplified from genomic DNA (Fig. 1).

Luciferase reporter assays demonstrated that a 5′ flanking region of the human *DPYD* gene (−1848 to −21) was able to initiate and up-regulate transcription by 4-fold in cultured Hs766T cells ($P = 0.038$; Fig. 3). In contrast, this 5′ flanking region of the *DPYD* gene did not induce transcription in HepG2 cells (Fig. 3). Endogenous DPD activity is 10-fold higher in Hs766T cells (90 pmol/min/mg protein) than HepG2 cells (9 pmol/min/mg protein). The 5′ flanking region of the *DPYD* gene is not GC-rich and has a low content of CpG dinucleotides, suggesting that DNA methylation

FIG. 2. 5′ Flanking sequence of the human (AF214571), rhesus (AF216267) and cynomolgus (AF216268) monkey *DPYD* genes (−1848 to +1, numbered from the translation start site at +1). Nucleotides in the monkey *DPYD* genes are indicated below the human sequence: −, conserved between human and monkey; ●, absence of nucleotide in species indicated. Putative transcription factor binding sites in the mammalian genes are underlined. An AP-3 site, unique to the human gene is in bold. ICS (interferon consensus sequence) and γ-IRE are indicated with dotted lines. The previously published 5′UTR (HSO9178) is in bold.


AABS
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PEA3
γ-IRE
γ-IRE
AP-3
PEA3
γ-IRE
CCAAT(-)
NF1
Sp-1
PEA3/Ets-1
AP-3
TATA-box
PEA3(E1A-F)
ICS
PEA3
E2A
TATA-box
C/EBP
γ-IRE
C/EBP
H-APF-1
TATA-box
PEA3(E1A-F)
CCAAT(-)
γ-IRE
HNF-4
AP-2
PEA3/Ets-1
γ-IRE
γ-IRE
Sp-1
NRE-box
PEA3
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human
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cynomolgus
-1771
-1693
-1616
-1538
-1460
-1382
-1304
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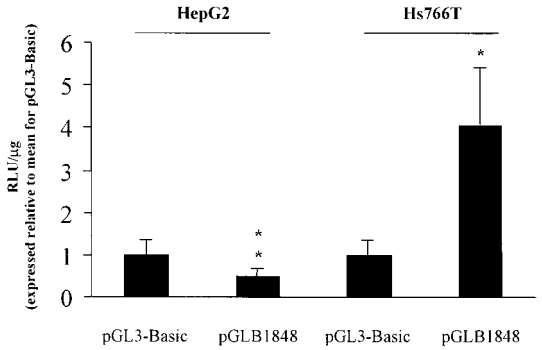

FIG. 3. Transcriptional activity of a 5′ flanking region of the human *DPYD* gene. pGL3-basic luciferase reporter vector and a clone containing a 5′ flanking region (−1848 to −21) of the *DPYD* gene inserted in to the MCS of the pGL3-basic vector [pGLB1848] were transiently transfected into Hs766T or HepG2 cells. Results are expressed relative to the mean RLU/μg for the pGL3-basic vector alone in each experiment and represent the mean ± SEM of 9 replicates from three separate transfections. * $P = 0.038$; ** $P = 0.007$.

is unlikely to play a key role in regulating *DPYD* transcription (25).

The 5′ flanking regions (−1824 to +1) of the rhesus (GenBank Accession No. AF216267) and cynomolgus (GenBank Accession No. AF216268) monkey *DPYD* genes had a high degree of homology with the human *DPYD* gene (>96%; Fig. 4). The 487 bp region between −525 and −39 had 100% identity in all three species. Computer analysis identified a TATA motif (−492) and an inverted CCAAT motif (−372) within this region (Fig. 2). Two other putative TATA boxes (−954 and −730) and an inverted CCAAT motif (−1516) were identified in the human and monkey *DPYD* genes. Multiple TFIID consensus sites were identified in the 5′ flanking region of the *DPYD* gene, including one (−100 to −96) at the major transcription start site previously identified in human liver at −98 (22). No TATA or CCAAT boxes were identified proximal to this transcription start site.

Consensus binding sites for liver-enriched transcription factors involved in regulating hepatic-specific gene expression, were identified in the 5′ flanking region of the *DPYD* gene. These included putative HNF-4, C/EBP and H-APF-1 binding sites which were identified in the human and monkey genes (Fig. 2). An AABS motif, which binds C/EBP and HNF-1 in other hepatically expressed genes (23), was identified at −1841 in the human *DPYD* gene, a region not evaluable in the monkey genes. Eight elements that commonly act as enhancers and bind the hormone-responsive PEA3/Ets-1 family of transcription factors (24) were identified in the human and monkey *DPYD* genes. Consensus binding sites for ubiquitously expressed factors (Sp-1, E2A and NFI) as well as cell-type specific factors (AP-2 and AP-3) were also identified and these may play a role in regulation of *DPYD* transcription in mammals (Fig. 2). A number of putative interferon (IFN) *cis*-acting sites, including γ-IFN response elements (γ-IRE) and an IFN binding factor consensus sequence (ICSbf) were identified in the mammalian *DPYD* genes (Fig. 2).

The 1.85 kb 5′ flanking region of the *DPYD* gene was sequenced in cancer patients with reduced ($n = 23$) or normal ($n = 14$) PBMC DPD activity. A total variant allele frequency of 4.1% (3/37) was found in this cancer population. Two heterozygous polymorphisms were identified (T-1590C and A-1378G, numbered from the translation start site at +1) in a patient with reduced DPD activity (137 pmol/min/mg protein) who experienced severe haematopoietic toxicity in response to 5FU therapy. The mutation at −1590 disrupts a putative γ-IRE that is conserved in mammals, as well as an AP-3 consensus sequence unique to the human *DPYD* gene (Fig. 2). This mutation (T-1590C) created an RFLP and was further analysed in 100 blood donors (Fig. 5). The T-1590C mutation was not detected in this sample, indicating that it is a rare variant with a frequency of <0.5% in healthy populations. The mutation at −1378 and a third heterozygous mutation (G-1345A), detected in a cancer patient with normal DPD activity (276 pmol/min/mg protein), did not disrupt any putative *trans*-acting sites.

The 5′ flanking sequence of the human *DPYD* gene did not have significant homology with any sequences in the EMBL/GenBank databases. However, comparison of the human *DPYD* sequence with EST databases identified three human mRNAs with homology to the 5′ flanking region of the *DPYD* gene. These transcripts included a full length human brain mRNA (4068 bp; Accession No. AB020687) that included a 489 bp region

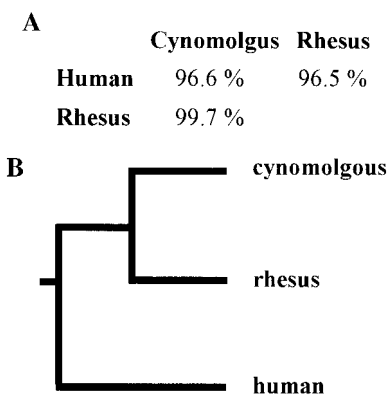

FIG. 4. Comparison of species differences in a 5′ flanking region (−1824 to +1) of the *DPYD* gene in human (AF214571), rhesus monkey (AF216267) and cynomolgus monkey (AF216268). GenBank accession numbers are shown in parentheses. (A) Degree of homology between the three species, (B) Phylogenetic tree demonstrating the divergence of the human and monkey *DPYD* genes (−1824 to +1), calculated using DNA parsimony algorithm, v3.57c in PIE at www.hgmp.mrc.ac.uk. The distance between the branches, calculated using Neighbor-Joining/UPGMA method v3.57c, was rhesus 0.00165; cynomolgus 0.00165; human 6.05835.

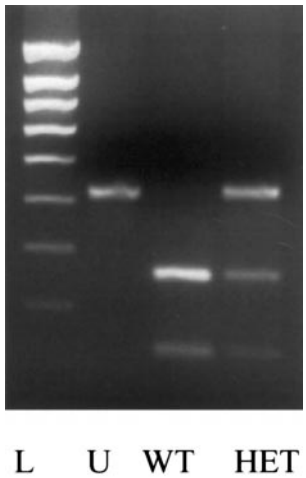

FIG. 5. PCR-RFLP analysis of a mutation detected at −1590 in the human *DPYD* gene. L, 100 bp DNA ladder; U, uncut; WT, T/T homozygous wild type, HET; T/C heterozygous mutant.

(3569 to 4068) with 100% homology to a *DPYD* 5′ region (−580 to −92). This mRNA is expressed on the opposite strand to that encoding DPD. Two human IMAGE clones from glioblastoma (437 bp, accession number AI419433) and uterus carcinoma (510 bp, Accession No. AI887596) had 100% homology with 5′ flanking regions of the *DPYD* gene (−728 to −303 and −877 to −368, respectively) and were transcribed from opposing strands.

DISCUSSION

This study describes the first investigation of transcriptional control of the pyrimidine catabolic pathway, through the cloning and functional characterisation of the *DPYD* gene promoter. DPD plays a key role in the metabolism of pyrimidines and pyrimidine-based antimetabolites in both normal and neoplastic tissues. Further insight into the mechanisms controlling DPD expression *in vivo* may identify novel molecular targets either to modulate existing pyrimidine based therapies, such as 5FU, or to develop more effective anti-proliferative agents.

A 1.83 kb 5′ flanking region upstream of the translation start site in exon 1 of the *DPYD* gene was sufficient to initiate and up-regulate transcription of a reporter gene in cultured pancreatic carcinoma cells. The relatively weak promoter activity (4-fold) is consistent with *in vivo* and *in vitro* transcription data for this low abundance transcript (16, 18). *DPYD* promoter activity was not evident in cultured HepG2 cells, which have almost undetectable endogenous DPD activity. These data indicate that down-regulation of DPD catalytic activity in cell culture (18) may be mediated at a transcriptional level.

Comparison of the 5′ flanking region of the *DPYD* gene in three mammalian species revealed that the entire 1.85 kb region upstream of the translation start site has been highly phylogenetically conserved. A region immediately upstream of the translation start site (−525 to −39) has complete identity in all 3 species, suggesting that this region is functionally important. Although a number of TATA and inverted CCAAT motifs were identified, none were found proximal to the major transcription start site previously identified in human liver at −98 (22), suggesting these TATA motifs may not be functional elements in the *DPYD* gene. However, multiple TFIID consensus sequences were identified, including one at the previously identified major transcription start site. TATA-less promoters can recruit TFIID for transcription initiation via an initiator element or to multiple transcription start sites in initiatorless promoters via an undefined mechanism (26, 27). The human *DPYD* gene may have one of these classes of promoters.

Partial mRNAs with 100% homology to 5′ flanking regions of the human *DPYD* gene were identified in the EST databases. This suggests that the *DPYD* gene, which has large unanalysed intronic regions and encompasses at least 950 kb (22), may be part of a highly complex bidirectional locus encoding multiple genes. This type of loci has been previously described for the 60 kb HLA locus which contains 13 overlapping transcription units (28). The apparently highly complex nature of the *DPYD* locus may provide further insight into the molecular mechanisms associated with neurological abnormalities in DPD-deficient paediatric patients. Many studies have described clinically normal, but DPD-deficient family members of neurologically affected paediatric patients (29, 30). The paediatric patients themselves also display highly variable clinical phenotypes (12). Identification of other transcription units within the *DPYD* locus, at least one of which is expressed in human brain, suggests that other gene/s may either mediate or contribute to the clinical phenotype of this inherited metabolic disorder.

Tissue-specific DPD expression may be mediated at a transcriptional level. DPD protein and catalytic activity are high in human liver and PBMC compared to other tissues (15). DPD mRNA levels are also much higher in liver than colon (16). Putative binding sites for liver-enriched transcription factors that have been shown to regulate the expression of other hepatically expressed genes (31) were identified in the *DPYD* gene. Conservation of these sites in human and monkey indicates that tissue-specific expression of DPD protein may be controlled, at least in part, by a transcriptional mechanism involving factors such as HNF-4, HNF-1, C/EBP and/or H-APF-1 in mammals. C/EBP transcription factors are enriched in monocytes as well as hepatocytes, the cells with the highest DPD activity *in vivo* (15, 32).

DPD expression may be modulated by cytokines such as IFN, as a number of putative γ-IRE were identified in the *DPYD* gene. Although there are no reports on the actions of γ-IFN on DPD expression, α-IFN and γ-IFN both increase 5FU cytotoxicity *in vitro* (33). In addition, α-IFN has been shown to down-regulate DPD catalytic activity in cell lines (34). Therapy with α-IFN in combination with 5FU is associated with reduced DPD activity and 5FU clearance in cancer patients (35). The presence of multiple IFN responsive elements in the *DPYD* gene, suggests IFN-mediated regulation of DPD activity may be via a transcriptional mechanism. A polymorphism that disrupts a putative AP-3 site and a putative γ-IRE was identified in our patient with reduced DPD activity and 5FU intolerance, consistent with a clinically relevant role for IFN in regulating *DPYD* transcription.

Complete sequencing of the 5′ flanking region of the human *DPYD* gene in a cancer population that included reduced and normal DPD activity individuals determined a variant allele frequency of 4.1%. This analysis demonstrated that in most cases (over 90%), reduced DPD activity is not explained by variant *cis*-acting elements in a 1.85 kb 5′ flanking region of the *DPYD* gene. Other mechanisms, such as variant distal *cis*-elements or differential expression of *DPYD*-associated binding proteins or other *trans*-acting factors may play a role in regulating DPD phenotype *in vivo.* Further characterisation of the *cis*-elements and *trans*-acting factors regulating *DPYD* transcription will provide insight into regulation of pyrimidine catabolism in normal and neoplastic tissues and may provide novel avenues for the development of improved anti-proliferative therapies.

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