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Cytochrome P450 1B1 mRNA Untranslated Regions Interact to Inhibit Protein Translation

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CYP1B1 mRNA is expressed constitutively in all normal extrahepatic human tissues, though the protein is usually undetectable. In contrast, CYP1B1 protein is expressed at high levels in tumors. In this study CYP1B1 mRNA and protein expression was measured in a panel of cell lines indicating that CYP1B1 regulation is altered in tumor cell lines in vitro. Interrogation of ONCOMINE revealed that CYP1B1 mRNA is not significantly overexpressed in tumors compared to normal tissues, suggesting CYP1B1 is subject to posttranscriptional control. Analysis of the CYP1B1 mRNA revealed a complex 5′ untranslated region (UTR) containing a small upstream open-reading frame (uORF). These features are present in mRNAs subject to translational control so the effect of the 5′UTR was tested using in vitro translation in CHO-K1 cells. The 5′UTR significantly inhibited luciferase reporter gene translation, and mutation of the uORF start codon abolished the inhibitory effect. The 5′UTR also interacted with the microRNA-27b recognition element in the CYP1B1 mRNA 3′UTR to almost completely inhibit translation. CYP1B1 is subject to a high degree of translational control, which may explain the absence of protein expression in normal cells. Alterations in translational control during malignant transformation may help to explain the tumor-specific expression of CYP1B1 protein. Mol. Carcinog. © 2009 Wiley-Liss, Inc.

Key words: CYP1B1; uORF; untranslated region; microRNA-27b; translation

INTRODUCTION

In normal tissues, cytochrome P450 1B1 (CYP1B1) is thought to play a role in the metabolism of endogenous molecules such as 17β-estradiol, melatonin, and retinoic acid [1–3]. Humans with CYP1B1 knockout mutations develop primary congenital glaucoma [4]; although the exact role of CYP1B1 in eye development and other normal tissues is unclear. The enzyme activates a number of exogenous carcinogens including benzo[a]pyrene (B[a]P) and may be responsible for the activation of the polycyclic aromatic hydrocarbon 7,12-dimethylbenz(a)anthracene (DMBA) as CYP1B1 null mice are resistant to DMBA-induced lymphomas [5,6]. CYP1B1 is the major producer of 4-hydroxyestradiol from 17β-estradiol [2]. 4-Hydroxyestradiol is thought to be an endogenous carcinogen as it undergoes redox cycling leading to accumulation of DNA mutations and is a more potent mitogen than 17β-estradiol [7]. In support of the evidence that CYP1B1 plays a role in carcinogenesis, single-nucleotide polymorphisms in the CYP1B1 gene are linked to increased risk of cancer, including prostate, endometrial, and ovarian cancer [8–10].

The pattern of human CYP1B1 expression is not typical of other cytochrome P450s. Unlike most cytochrome P450s, CYP1B1 mRNA is not expressed in the liver but is detected at high levels in extrahepatic tissues [11]. CYP1B1 protein is generally undetectable in normal extrahepatic tissues; however, the protein is expressed at high levels in a wide variety of tumor types [12,13]. Expression of CYP1B1 protein has also been observed in premalignant prostate lesions and cervical intraepithelial neoplasia [14,15]. We have also demonstrated that CYP1B1 is expressed in esophageal hyperplasia in a rat model of gastric and duodenal reflux [16].

In normal cells CYP1B1 mRNA expression is regulated by the aromatic hydrocarbon receptor (AhR) complex which binds to dioxin responsive elements within the CYP1B1 promoter [17]. The CYP1B1 promoter also contains a nonconsensus estrogen responsive element [18].

The factors controlling the differential regulation of CYP1B1 expression in normal and tumor tissues are poorly characterized. CYP1B1 mRNA is not overexpressed in matched normal and tumor

Abbreviations: CYP1B1; cytochrome P450 1B1; AhR; aromatic hydrocarbon receptor complex; UTR; untranslated region; Q-PCR, quantitative real-time polymerase chain reaction; uORF; upstream open-reading frame.

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samples from human kidney or breast [19,20], but it is overexpressed in some prostate tumor tissues compared to normal tissues [21]. The CYP1B1 mRNA 3′ untranslated region (UTR) contains a microRNA-27b recognition element which can repress translation of reporter gene constructs in the presence of microRNA-27b expression [22]. A number of proteins which are commonly overexpressed in tumors compared to normal tissues have translational control elements within their mRNA 5′UTRs [23].

In order to provide further information on CYP1B1 mRNA expression in immortalized tumor cell lines, a survey of CYP1B1 mRNA and protein expression was performed. To indicate the importance of translational control in the tumor-specific expression of CYP1B1 protein, ONCOMINE was interrogated to determine the relative expression of CYP1B1 mRNA in normal and tumor tissues. The CYP1B1 mRNA 5′UTR was analyzed to identify other elements which could be responsible for translational control, and the effect of these elements was investigated in combination with the 3′UTR microRNA-27b recognition element.

MATERIALS AND METHODS

Cell Culture and Reagents

MCF-7, MDA-MB-231, MDA-MB-468, HT-116, and MBR-62 cells were maintained in DMEM with 10% fetal calf serum; HT-29 and RKO cells in MEM with 10% fetal calf serum; T24 cells in McCoys medium 5A with 10% fetal calf serum; DU145 and PC3 cells in RPMI 1640 with 10% fetal calf serum; LNCaP cells in RPMI 1640 with 1 M HEPES; CHO-K1 in F-12 (Ham) with 10% fetal calf serum. All reagents were obtained from Invitrogen (Paisley, UK).

Quantitative Real-Time PCR (Q-PCR)

Total cellular RNA was isolated from 80% confluent cultures using TRIzol reagent (Invitrogen), and 2 μg was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen). Quantitative real-time PCR (Q-PCR) was performed on the Lightcycler ® 2.0 System (Roche, Welyn Garden City, UK) in 20 μL reactions with 10 μL of Quantitect SYBR Green PCR Master Mix (Qiagen, Crawley, UK), 2 μL of undiluted cDNA, and 10 μM primers: CYP1B1, 5′-AACGTACCGGCCACTATCAC and 5′-CCACGACCTGATCCAATTCT; hypoxanthine–guanine phosphoribosyltransferase (HPRT), 5′-GACCAGTCAACAGGGACAT and 5′-AAGCTTGCGACCTTGACCAT. Q-PCR was performed at 95°C for 900 s followed by 35 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Melting curve analysis was performed to ensure the absence of nonspecific PCR products. Data were exported to Relative Quantification Software Version 1.0 (Roche), and the HPRT-normalized expression of CYP1B1 mRNA in each cell line, relative to MCF-7 cells, was determined using efficiency correction calculation.

Western Blotting

Eighty percent confluent cultures of cells were scraped into chilled lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate with protease inhibitors). Thirty micrograms of protein was subjected to Western blotting using standard protocols with antibody incubations at room temperature for 3 h. Rabbit anti-CYP1B1 antibody (Gentest/BD Biosciences, Franklin Lakes, NJ; 1:1000) or mouse anti-CYP1B1 antibody (kind gift of G. Murray, University of Aberdeen, UK; 1:1000) were incubated with rabbit anti-β-actin antibody (Sigma, Gillingham, UK; 1:10 000) and detected using horse radish peroxidase-conjugated antirabbit antibody (Gentest; 1:5000) or mouse horse radish peroxidase-conjugated antimouse antibody (Sigma, Kent, UK; 1:15 000) with SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Cramlington, UK).

ONCOMINE Analysis

Cancer versus normal microarray data sets containing data on CYP1B1 were retrieved from the ONCOMINE database at www.oncomine.org [24]. The difference in CYP1B1 expression between normal and tumor tissues was considered significant if the *q*-value (two sided *t*-test corrected for the method of false discovery rate) was less than 0.05.

5′ Untranslated Region Analysis

The CYP1B1 was scanned for uORFs using the NCBI ORF Finder tool (available at http://www.ncbi.nlm.nih.gov/projects/gorf/). Energy of unwinding was calculated using MFOLD selecting the linear and RNA options [25]. The homology between the human, rat, and mouse 5′UTRs was calculated using ClustalW [26]. mRNAs were classed into their predicted method of translational control according to Davuluri et al. [27].

Plasmid Construction

Genomic DNA was isolated from MCF-7 cells [28] and the full-length 373 bp CYP1B1 5′UTR was amplified by PCR using Taq DNA polymerase (Invitrogen) using the forward 5′-CTTTTGCAAAAAGCTACTCTGGAGTGGGAGTGGGAG and reverse 5′-TTGGCGTCTTCCATGGTGCGGGGAGGTGCGGTTT primers. pGL3 promoter plasmid (hereafter called pLuc) (Promega Corp., Madison, WI) was digested using *Nco*I and *Hin*dIII and ligated to the CPY1B1 5′UTR using the In-Fusion Dry Down PCR cloning kit (Clontech, Mountain View, CA) to create p5-Luc. p5mut-Luc was created from p5Luc by standard site directed mutagenesis protocols using Platinum Pfx DNA polymerase (Invitrogen) with the forward 5′-CGTCAATTCCCAAGCCCTTGCGGCT-

CTC and reverse 5′-GAGAGCCGCAAGGGCTTGG-GAATTGACG primers. A 120-bp fragment (+4311 to +4439) containing three copies of the microRNA-27b recognition element, and a control 128 bp fragment (+3899 to +4109) from the CYP1B1 3′UTR [22] were amplified by PCR from genomic DNA using the primers to the microRNA-27b region; forward 5′-TTTCTAGATGTCTCAGGTTTGTTTT and reverse 5′-GAATCTAGAATGCAACTATTTGAT-CT, control region forward 5′-GCTCTAGATGCCTCAT-TATGTCAACCA and reverse 5′-GCTCTAGACC-TTACCTTTCTTCCATATAAA, and cloned into the pLuc, p5-Luc, or p5-Luc plasmids downstream of the CYP1B1 ORF using *Xba*I. The cloning and site-directed mutagenesis was confirmed by sequencing for all plasmids.

In Vitro Translations

PCR was used to generate DNA fragments incorporating the T7 promoter sequence. Plasmid DNA was amplified using forward 5′-TAATACGACTCAC-TATAGGGTGGTAAAGCCACCATGGAAGA for pLuc, 5′-TAATACGACTCACTATAGGGGCTACTCTGGA-GTGGGAGTGG primers for p5-Luc and p5mut-Luc, and reverse 5′-CCCCCTGAACCTGAAACAT primer using Taq DNA polymerase (Invitrogen). PCR fragments were extracted with phenol/chloroform/isoamyl alcohol, resuspended in water, and 0.5 μg of DNA was in vitro transcribed using T7 Cap-Scribe (Roche) at 37°C for 1 h. RNA (0.5 μg) was in vitro translated using rabbit reticulocyte lysate (nuclease treated, Promega Corp.) for 90 min at 30°C. In some experiments, RNA was heat denatured by incubation at 65°C for 10 min and immediately placed on ice for 2 min before in vitro translation. Firefly luciferase assays were performed in triplicate on 2 μL of each in vitro translation using the Dual Glo Luciferase™ Assay System (Promega Corp.). In each experiment, the mean luciferase activities were expressed as a percentage of the mean luciferase activity in p5Luc. Results were assessed using unpaired two-tailed *t*-tests (95% confidence interval) using Prism 3.0 (GraphPad, San Diego, CA).

Transient Transfections

3×10^4 CHO-K1 cells were plated per well of a 96-well plates 18 h before transfection to obtain approximately 80% confluency at transfection. Fifty nanograms of pLuc plasmid DNA and 40 ng of pRL-TK control plasmid were cotransfected using Lipofectamine 2000 (Invitrogen). Luciferase activities were assayed at 24 h using Dual Glo Luciferase Assay System (Promega Corp.) and adjusted to values obtained in untransfected control cells. The firefly/renilla ratio was expressed as a percentage of the firefly/renilla ratio in cells transfected with pLuc. Results were assessed using unpaired two-tailed *t*-tests using Prism 3.0 (GraphPad).

RESULTS

CYP1B1 mRNA and Protein Expression in Immortalized Cell Lines

There are limited data available on the relative expression of CYP1B1 mRNA and protein in immortalized human cells lines. Q-PCR was performed in triplicate on cDNA generated from a variety of cell lines to determine CYP1B1 mRNA expression. Melting curve analysis confirmed a single PCR product in each reaction. In HMEC, MBR-62, RKO, T24, UMUC-3, or LNCaP cells CYP1B1 mRNA was not detectable. In the remainder of cell lines tested there was a variable expression of CYP1B1 (Fig1A). The expression pattern of CYP1B1 was not dependent on the origin of the cell line, tumor stage, or grade. CYP1B1 protein expression was analyzed by Western blotting using two anti-CYP1B1 antibodies (Figure 1B). Despite loading 30 μg of cell lysate and using antibodies capable of detecting picomolar quantities of CYP1B1, the protein was not detected in any of the cell lines tested using the mouse monoclonal antibody (G. Murray). An immunoreactive band close to the molecular weight of CYP1B1 was observed using the Gentest polyclonal antibody; however, this is a nonspecific band as it was detected in all cells including those which do not express CYP1B1 mRNA (data not shown). As a result, studies using the Gentest antibody should be interpreted with caution.

CYP1B1 mRNA Expression Is Not Significantly Overexpressed in Tumors Compared to Normal Tissues

ONCOMINE database is a repository of microarray data from multiple studies comparing tumor and normal tissues. The database was interrogated to determine if the tumor-specific expression of CYP1B1 protein is due to elevated mRNA expression in tumors. Information was available on the relative expression of CYP1B1 mRNA in 52 microarrays comparing tumor and normal tissue from 17 different organ sites (Table 1). In the majority of the microarrays (76.9%), there was no significant difference in CYP1B1 mRNA expression between normal and tumor tissues. CYP1B1 mRNA was significantly overexpressed in tumors in 11 studies (21.2%), and under expressed in tumors in 1 study compared to normal tissues (1.9%). These results indicate that the tumor-specific expression of CYP1B1 is unlikely to be due to alterations in transcriptional control.

Translational Control Elements Are Present in the CYP1B1 mRNA 5′UTR

The features of the CYP1 family mRNAs were analyzed to determine if the mRNA 5′UTR could exert translational control (Table 2, Fig2). Both human and rodent CYP1B1 mRNA contain long complex 5′UTRs compared to human CYP1A1 and

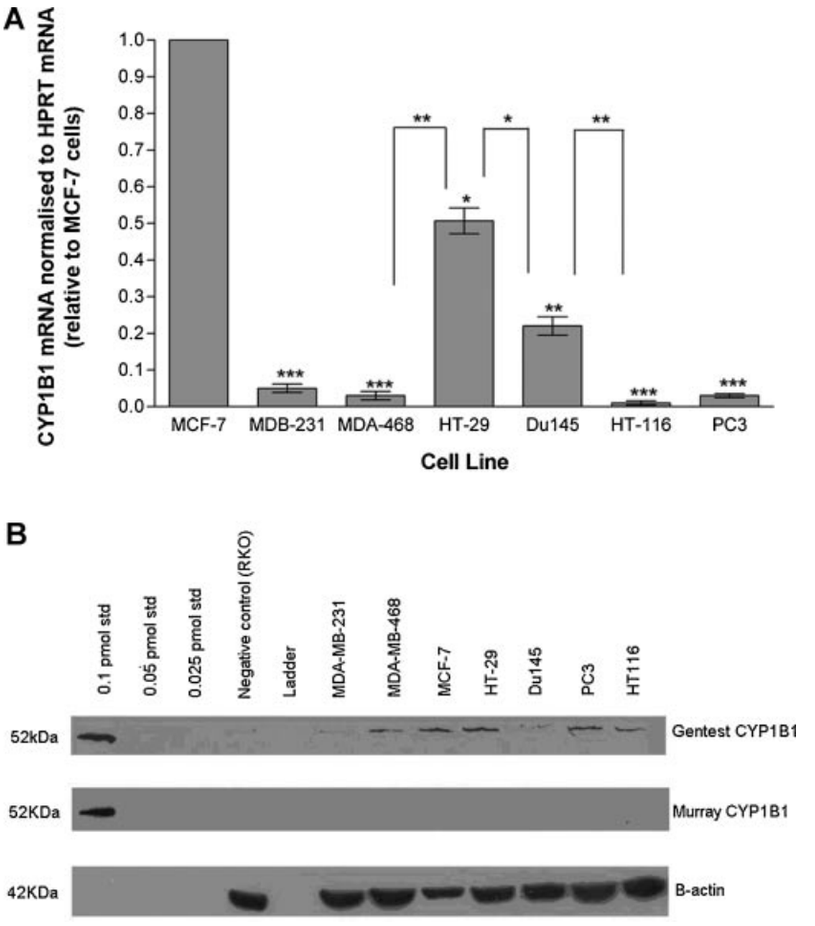

Figure 1. CYP1B1 mRNA and protein expression in immortalized cell lines. (A) CYP1B1 mRNA expression in cell lines was measured in triplicate by Q-PCR, normalized to HPRT mRNA. No CYP1B1 mRNA was detected in RKO, LNCaP, HMEC, MBR-62, or UMUC-3 cells. Two-tailed unpaired *t*-tests: $***P \leq 0.0001$, $**P \leq 0.005$, $*P \leq 0.05$. (B) Thirty micrograms of total cell lysates and CYP1B1 standard were subjected to Western blotting using polyclonal (Gentest) and monoclonal (Murray) CYP1B1 antibodies. Representative β-actin (loading control) results are shown.

CYP1A2 mRNA. MFOLD analysis revealed that both the human and rodent CYP1B1 mRNA 5′UTRs are predicted to fold into complex secondary structure with high energies of unwinding. The human CYP1B1 5′UTR also contains a small upstream open-reading frame (uORF) encoding a 16-amino-acid peptide (MGIDATHRPPVSISTL) with a molecular weight of 1.6 kDa and predicted p*I* of 6.5, which is not present in the rodent CYP1B1 or human CYP1A mRNAs. As the human CYP1B1 has a long complex 5′UTR containing a high degree of secondary structure and an uORF, CYP1B1 mRNA is predicted to be regulated by translational control whereas the CYP1A mRNAs are predicted to be translationally unregulated [27].

CYP1B1 5′UTR Exerts Translational Control Via a Small uORF

To test the effect of the CYP1B1 mRNA 5′UTR on translation, the 5′UTR was cloned into luciferase reporter plasmids (Fig3A). RNA was in vitro transcribed and equal amounts of RNA were in vitro translated in three separate experiments (Fig3B). The presence of the CYP1B1 5′UTR in p5-Luc-derived RNA (ii) significantly inhibited luciferase activity to 25% of the pLuc control activity (i, $P < 0.001$, Figure 3B) confirming the theoretical predictions that CYP1B1 mRNA 5′UTR is capable of exerting translational control. The luciferase activity of RNA generated from p5mut-Luc (iii), where the uORF was abolished by mutating the uORF start codon, was not significantly different to the luciferase activity of control pLuc RNA, suggesting that the ability of the CYP1B1 5′UTR to exert translational control is mediated by the uORF. As the CYP1B1 5′UTR contains a high degree of secondary structure, the RNA was heat denatured and in vitro translated. Using heat-denatured RNA did not significantly affect the luciferase activities of either the p5-Luc or p5mut-Luc reactions, indicating that secondary

Table 1. Relative CYP1B1 mRNA Expression in Human Tumor Samples Compared to Nontumor Tissues From Microarray Studies in ONCOMINE

Tissue	Tumor compared to normal	Change	Normal tissue (number of samples)	Tumor tissue (number of samples)
Lymph	Overexpressed	8.50	Blood CD19+ B-cell (6) and germinal center B cells (6)[a]	B-cell lymphoma (274)[b]
	No change	—	CD19/B cells (31)[a]	B-cell lymphoma (68)[b]
	No change	—	CD19/B cells (31)[a]	CLL (12)[c]
	No change	—	CD19/B cells (31)[a]	Follicular lymphoma (9)
	Underexpressed	0.70	B cells (7), plasma cells (37)[a]	Multiple myeloma (74)
Brain	Overexpressed	1.74	Normal white matter (7)	Glioblastoma (25)
	No change	—	Normal temporal lobe (6)	Glioma (45)
	No change	—	Postmortem normal brain (3)	Glioblastoma (30)
	No change	—	Normal white matter (3)	Astrocytoma (8)
	No change	—	Normal meninges (3)[d]	Meningioma (15)
Salivary gland	Overexpressed	2.15	Normal (6)	Adenoid carcinoma (16)
Head and neck	No change	—	Normal (2), tumor adjacent (2)	SCC (34)
	No change	—	Normal tonsil (3)	SCC (55)
Thyroid	No change	—	Tumor adjacent (8)	Carcinoma (8)
Lung	Overexpressed	8.17	Tumor adjacent (6)	SCC (13)
	Overexpressed	1.68	Tumor adjacent (17)	Adenocarcinoma (139)
	Overexpressed	11.56	Tumor adjacent (6)	Adenocarcinoma (40)
	No change	—	Tumor adjacent (10)	Adenocarcinoma (86)
	No change	—	Tumor adjacent (11)	Adenocarcinoma (11)
	No change	—	Tumor adjacent (17)	SCC (17)
	No change	—	Tumor adjacent (17)	Small cell (6)
	No change	—	Tumor adjacent (6)	Small cell (4)
	No change	—	Tumor adjacent (6)	Large cell (4)
	No change	—	Tumor adjacent (7)	Carcinoid (20)
Adrenal	No change	—	Normal (3)	Adenocarcinoma (11)
Colon	No change	—	Tumor adjacent (18)	Adenocarcinoma (18)
Pancreas	No change	—	Tumor adjacent (5)	Adenocarcinoma (12)
	No change	—	Duct cells (6)	Ductal carcinoma (8)
	No change	—	Duct cells (25)	Ductal carcinoma (24)
	No change	—	Normal (5)	Adenocarcinoma (10)
	No change	—	Tumor adjacent (11)	Tumor epithelia (14)
Kidney	No change	—	Tumor adjacent (3)	Clear cell carcinoma (26)
	No change	—	Tumor adjacent (9)	Clear cell carcinoma (9)
Bladder		—	Normal (4)	Carcinoma (67)
Liver	No change	—	Benign disease (7)	Carcinoma (104)
	Underexpressed	0.002	Tumor adjacent (76)	Carcinoma (104)
Breast	No change	—	Normal (3), fibroadenoma (1)	Ductal carcinoma
	No change	—	Normal (3), fibroadenoma (1)	Lobular carcinoma
	No change	—	Normal (4)	Carcinoma (7)
Endometrium	No change	—	Normal (4)	Adenocarcinoma (10)
Ovary	No change	—	Normal (4)	Adenocarcinoma (28)
	No change	—	Normal (3)	Adenocarcinoma (31)
	No change	—	Normal epithelia (4)	Tumor (6)
Testis	Overexpressed	$3.6E^{-8}$	Normal (6)	Germ cell tumor (91)
	No change	—	Normal (14)	Seminoma (23)
Prostate	Overexpressed	1.85	Tumor adjacent (3)	Carcinoma (23)
	Overexpressed	1.80	Tumor adjacent (50)	Carcinoma (52)
	No change	—	Nontumor prostate (23)	Carcinoma (64)
	No change	—	Tumor adjacent (6) and BPH (16)	Carcinoma (59)
	No change	—	Tumor adjacent (9)	Carcinoma (25)
	No change	—	Tumor adjacent (4)	Carcinoma (8)
	No change	—	Tumor adjacent (15)	Carcinoma (15)
	No change	—	Tumor adjacent (41)	Carcinoma (62)

[a]Purified in vitro.
[b]Diffuse large cell.
[c]Chronic lymphocytic.
[d]Postmortem.

Table 2. Analysis of the 5′ Untranslated Regions of the CYP1 Family mRNAs

	mRNA				
	CYP1B1			CYP1A1	CYP1A2
	Human	Mouse	Rat	Human	Human
Accession number	NM_000104	NM_009994	NM_012940	NM_000499	NM_000761
Homology to human CYP1B1	—	65%	65%	—	—
5′UTR length (nucleotides)	373	357	372	123	65
5′TOP	No	No	No	No	No
Overall energy of unwinding (Kcal/mol)	−147.0	−119.4	−139.73	−22.15	−7.2
uORFs	1	0	0	0	0
Classification	Class I, poorly translated	Class I, poorly translated	Class I, poorly translated	Class III, translationally unregulated	Class III, translationally unregulated

5′TOP, terminal oligopyrimidine tract; uORF, upstream open reading frame.
The 5′UTR mRNA sequences were analyzed as described in the Materials and Methods Section and classified by their likely method of translational control [27].

structure within the CYP1B1 5′UTR does not have an effect on translational control (Figure 3B).

To further test the effect of the CYP1B1 mRNA 5′UTR in a cell-based assay, the plasmids were transiently transfected into CHO-K1 cells with a control plasmid expressing renilla luciferase. Twenty-four hours later, firefly luciferase activities were assayed and normalized to renilla activity to account for variations in transfection efficiency and expressed as a percentage of the parental pLuc plasmid (Figure 3C). As observed in the in vitro translations, the CYP1B1 5′UTR (p5-Luc, ii) significantly reduced luciferase activity to 53% of the control luciferase pLuc activity (i, $P < 0.0001$);

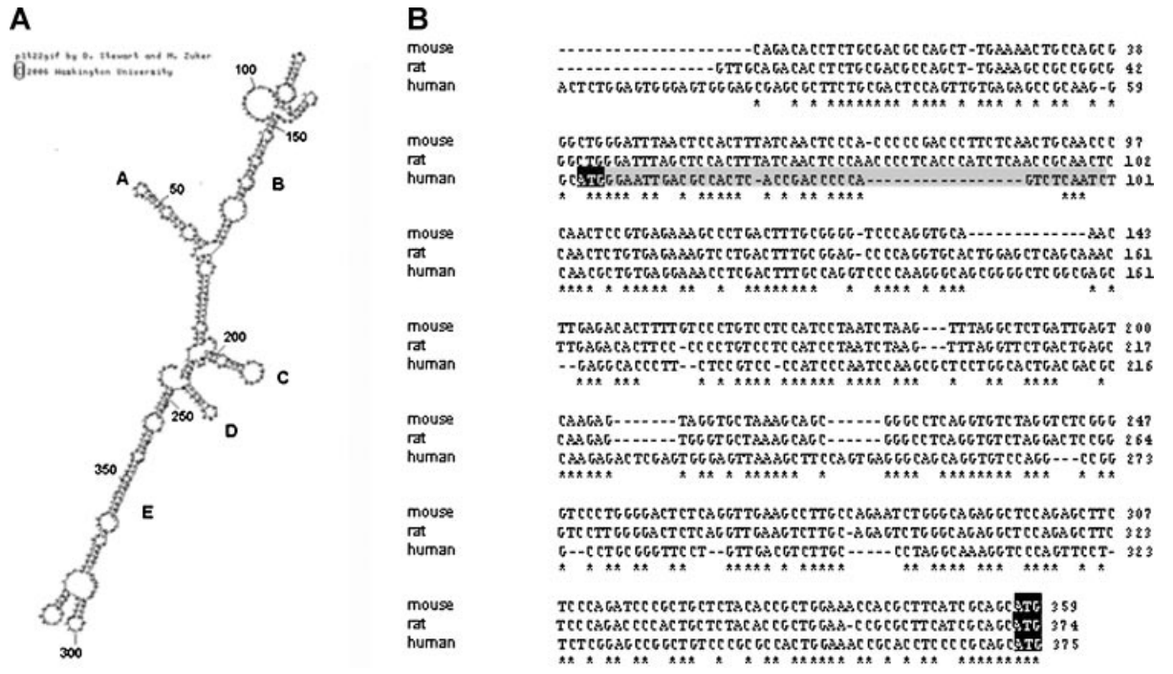

Figure 2. Features of the CYP1B1 mRNA 5′UTR. (A) Predicted secondary structure of the human CYP1B1 mRNA 5′UTR. (B) Alignment of the human, mouse, and rat CYP1B1 mRNA 5′UTR regions with start codons highlighted (black) and the human uORF (gray) indicated.

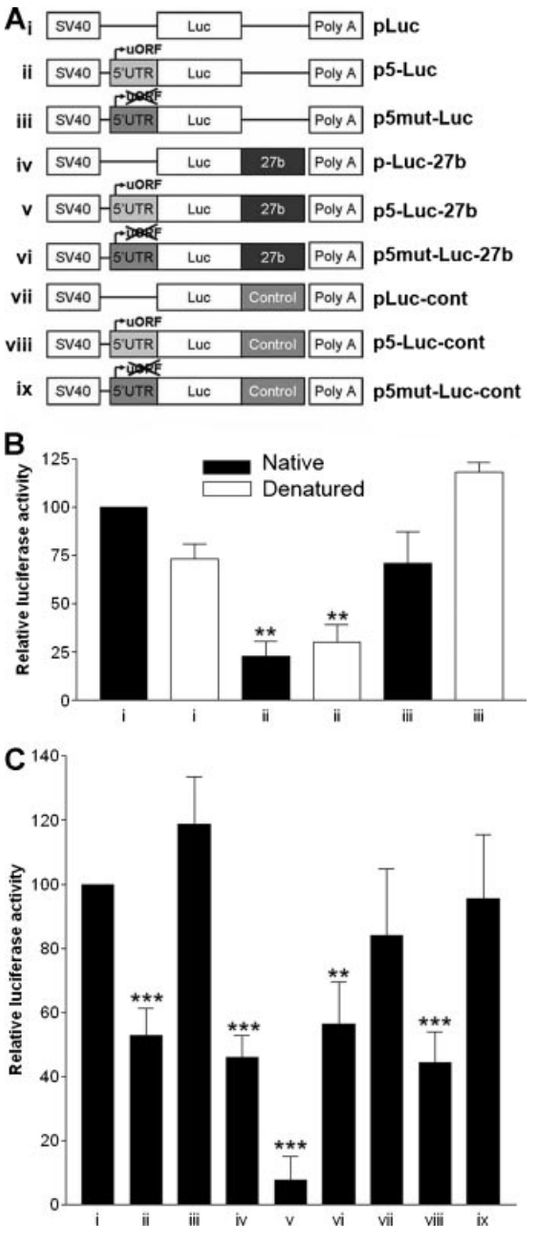

Figure 3. Effect of the CYP1B1 5′UTR and 3′UTR microRNA-27b recognition element on translation. (A) Schematic representation of luciferase reporter plasmids used in the study. The full-length 373 bp CYP1B1 5′UTR was cloned so the luciferase start codon replaced the CYP1B1 start codon. The microRNA-27b recognition element (128 bp) and control region (120 bp) were inserted downstream of luciferase (B) pLuc and the p5-Luc plasmids were in vitro transcribed, equal amounts of native or heat denatured RNA were used to program rabbit reticulocyte in vitro translations and luciferase activity was assayed after 90 min. (C) Effect of the CYP1B1 5′UTR and 3′UTR microRNA-27b-binding element on luciferase reporter translation in CHO-K1 cells. Forty-eight hours later firefly luciferase activity was assayed and normalized to renilla luciferase activity. Two-tailed unpaired *t*-tests: $***P \leq 0.0001$, $**P \leq 0.005$, $*P \leq 0.05$.

mutation of the uORF start codon in p5mut-Luc (iii) abolished this effect. These results indicate that the uORF in the CYP1B1 5′UTR is capable of exerting translational control in both in vitro translations and in cell based assays.

The CYP1B1 5′ and 3′UTR May Interact to Exert Translational Control

To investigate whether the CYP1B1 5′UTR uORF could interact with previously characterized translational control elements in the 3′UTR [22], luciferase reporter plasmids were constructed containing the CYP1B1 27b microRNA binding element or a control 3′UTR region and transfected into CHO-K1 cells (Figure 3A and C). The presence of the 3′UTR microRNA-27b recognition element (pLuc-27b, iv) leads to a significant decrease in luciferase activity to 46% of pLuc activity (i, $P < 0.01$). The inhibitory effect was not observed when the microRNA recognition element region was replaced by a control region from the CYP1B1 3′UTR (pLuc-cont, vii). When both the CYP1B1 5′UTR, containing the uORF, and the 3′UTR microRNA recognition element were present (p5-Luc-27b, v), luciferase activity was reduced to 8% of the activity in pLuc (i) reactions, $P < 0.0001$. When both the 5′ and 3′ regions were present (p5-Luc-27b, v) there was a significantly greater reduction in luciferase activity to 8% compared to either the uORF (p5-Luc, ii, 53%) or microRNA-27b recognition element alone (p-Luc-27b, iv, 46%, $P < 0.05$). This suggests that the uORF and microRNA-27b recognition element can interact to exert tighter control on translation than one of these elements alone. To test the specificity of this observation, either the 5′UTR uORF was mutated to remove the start codon (p5mut-Luc-27b, vi), or the microRNA recognition element was replaced with a control region from the 3′UTR (p5-Luc-cont, viii). In both cases, an increase in luciferase activity compared to p5-Luc-27b (v) was observed. The luciferase activity in these reactions was not significantly different to the activity in reactions with plasmids containing only the 5′UTR uORF (p5-Luc, ii) or only the microRNA recognition element (p-Luc-27b, iv). As the mutated uORF sequence had no effect on translation in the presence of the microRNA recognition element, and the control 3′UTR region had no effect on translational control exerted by the 5′UTR uORF, the synergistic effect of the uORF and microRNA recognition element on translation is likely to be due to an interaction of both elements.

DISCUSSION

CYP1B1 mRNA is expressed in all tissue types in humans [11]. The absence of constitutive CYP1B1 mRNA expression in extrahepatic tumor cell lines indicates that CYP1B1 mRNA is regulated differently in vitro, possibly due to the absence of necessary transcription factors or ligands of the AhR complex.

The AhR nuclear translocator (ARNT) can repress CYP1B1 translation in mouse liver cell lines in the absence of AhR ligands [29]. As CYP1B1 protein is expressed at high levels in a wide variety of human tumor types [12,13], the absence of CYP1B1 mRNA from some cell lines and the lack of detectable CYP1B1 protein in all tumor cell lines tested suggest CYP1B1 protein is also subject to differential regulation in vitro. It has previously been reported that CYP1B1 protein can only be detected in cell lines when induced by AhR ligands such as TCDD [30,31]. Our results suggest that studies of CYP1B1 mRNA and protein expression or induction in cell lines should be interpreted carefully as they may not reflect the response in vivo.

There have been few attempts to correlate CYP1B1 protein expression in tumors with a corresponding increase in mRNA compared to normal tissues; however, this is critical to understand the factors regulating CYP1B1 expression. One study indicated CYP1B1 mRNA was overexpressed in a single individual's prostate carcinoma compared to normal prostate [21]; however, others have reported that the mRNA is not overexpressed in breast or kidney tumors compared to matched normal tissues [19,20]. ONCOMINE microarray data reveal that CYP1B1 mRNA is not overexpressed in a large number of tumor types compared to normal tissues, suggesting that the differential expression of CYP1B1 protein in human tumors and normal tissues must be due to altered posttranscriptional control.

This led us to investigate if the CYP1B1 mRNA 5′UTR could exert translational control, as a number of proteins which are overexpressed in cancer are subject to altered translational control [23]. The human CYP1B1 mRNA was found to contain two features associated with posttranscriptional regulation, a long 5′UTR with extensive secondary structure and an uORF. Removing the RNA secondary structure by heat denaturing prior to use in in vitro translations had negligible effect on luciferase translation, indicating that the secondary structure in the 5′UTR does not play a significant role in CYP1B1 translation.

The uORF in the CYP1B1 mRNA 5′UTR was inhibitory to translation of a reporter mRNA in both in vitro translation reactions and cell-based assays. The ability of the CYP1B1 uORF to inhibit translation is comparable to the 30% reduction in translation observed when an artificial uORF was introduced into the 5′UTR of a reporter gene [32]. It has been reported that the majority of uORFs inhibit translation of the main ORF [33]. An uORF is not present in the rodent CYP1B1 mRNAs; however, it has been demonstrated that at least 30% of human uORFs are not present in rodents, suggesting their role is species specific [34].

The CYP1B1 uORF may affect translation via a number of mechanisms. The uORF start codon has a consensus Kozak sequence; therefore, it is likely to be recognized by scanning ribosomes [35]. If the uORF is translated, the scanning ribosome could dissociate after the uORF, preventing translation of CYP1B1 ORF. Alternatively, ribosomes may continue scanning after the uORF and translate CYP1B1 if they acquire new initiation factors such as Met-tRNA$_i$ [32].

It has previously been shown that the CYP1B1 mRNA 3′UTR contains a microRNA-27b recognition element which can also repress reporter gene and CYP1B1 translation [22]. We observed a synergistic interaction between the CYP1B1 uORF and microRNA recognition element which led to an almost complete repression of reporter gene translation. It is therefore likely that the absence of CYP1B1 protein in normal cells, where the mRNA is constitutively expressed, is due to the high degree of translational control exerted by the 5′ and 3′UTRs. It has been suggested that the presence of complex 5′UTRs and uORFs act as controls to ensure the levels of certain proteins, such as signaling molecules and receptors, are kept at low levels [32]. Repression of CYP1B1 protein via translational control may act to prevent accumulation of 4-hydroxyestradiol, the major product of CYP1B1 17β-estradiol metabolism [2], which is a carcinogen [7]. Regulation of CYP1B1 protein expression in normal cells may also be required to prevent inappropriate metabolism of other signaling molecules such as melatonin and retinoic acid [1,3].

mRNA molecules can circularize and both the 5′UTR and 3′UTR interact with translation initiation factors and other proteins involved in translation such as poly(A)binding protein which allows efficient initiation [36]. Similar to CYP1B1, Her-2/neu protein is overexpressed in a variety of tumor cells compared to normal cells, often in the absence of mRNA amplification or overexpression, and the mRNA contains a short uORF which represses Her-2 translation in normal cells. In the presence of the Her-2/neu 3′UTR, the inhibitory effect of the uORF is relieved in tumor cells. The Her-2 3′UTR is known to interact with a number of proteins and it is hypothesized that differential expression of these factors in tumors affects the ability of the 3′UTR to guide efficient reinitiation of scanning ribosomes at the Her-2 start codon after translation of the uORF, though the mechanism of this effect has not yet been fully elucidated [37].

The data presented support a role for the CYP1B1 5′UTR uORF and 3′UTR microRNA-27b recognition element in inhibition of CYP1B1 translation. In tumors, altered expression of factors, which interact with the uORF or microRNA recognition element, such as microRNA-27b, may explain the increased expression of CYP1B1 protein in these tissues. This is supported by evidence that increased expression of microRNA-27b correlates with a decreased

expression of CYP1B1 protein in human breast cancers [22].

Recently, there has been considerable research on SNPs in the coding region of CYP1B1 and their effect on the protein structure, metabolism of carcinogenic molecules, and odds-risks of developing cancer [8–10]. We have not investigated the effect that these polymorphisms have on the translational control mechanism presented in this manuscript. It is also possible that SNPs may exist in the 5′ or 3′UTRs of the CPY1B1 gene, which have been overlooked by researchers focusing on the protein coding sequence. It is important to study the effect of SNPs on the translational control of CPY1B1. It is possible that such polymorphisms may lead to a reduction in, or inhibition of CPY1B1 translational control, increasing CPY1B1 protein expression, and elevating cancer risk via the elevated production of carcinogenic metabolites.

In conclusion, this study has demonstrated that CYP1B1 expression in tumors is due to altered posttranscriptional control. Further investigation into this translational control mechanism may allow targeting of this process to inhibit tumor-promoting events in premalignant and tumor cells by preventing expression of CYP1B1 and inhibiting activation of carcinogenic molecules.

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