

Interaction of Heterogeneous Nuclear Ribonucleoprotein A1 with Cytochrome P450 2A6 mRNA: Implications for Post-Transcriptional Regulation of the *CYP2A6* Gene

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Received December 1, 2003; accepted February 23, 2004

This article is available online at <http://molpharm.aspetjournals.org>

ABSTRACT

The human xenobiotic-metabolizing enzyme cytochrome P450, *CYP2A6*, catalyzes the bioactivation of a number of carcinogens and drugs and is overexpressed in cases of liver diseases, such as cirrhosis, viral hepatitis, and parasitic infestation, and in certain tumor cells. This suggests that *CYP2A6* may be a major liver catalyst in pathological conditions. In the present study, we have addressed molecular mechanisms underlying the regulation of the *CYP2A6* gene. We present evidence of several proteins present in human hepatocytes that interact specifically with the 3'-untranslated region (UTR) of *CYP2A6* mRNA. Biochemical and immunological evidence show that the RNA-protein complex of highest intensity contains the heterogeneous nuclear ribonucleoprotein (hnRNP) A1 or a closely related protein. Mapping of the hnRNP A1 binding site within *CYP2A6* 3'-UTR reveals that the smallest portion of RNA sup-

porting significant binding consists of 111 central nucleotides of the 3'-UTR. Our studies also indicate that hnRNPA1 from HepG2 cancer cells exhibits modified binding characteristics to the *CYP2A6* 3'-UTR compared with primary hepatocytes. We found that the level of *CYP2A6* mRNA remains high in conditions of impaired transcription in primary human hepatocytes, showing that *CYP2A6* expression can be affected post-transcriptionally in conditions of cellular stress. Our results indicate that the post-transcriptional regulation involves interaction of the hnRNP A1 protein with *CYP2A6* mRNA. The present data suggest that hnRNPA1 is a critical regulator of expression of the human *CYP2A6* gene and support the notion that this P450 isoform may be of particular significance in stressed human liver cells.

The human cytochrome P450 (P450) *CYP2A6* is an inducible hepatic enzyme involved in the biotransformation of nicotine and several clinically used drugs such as halothane and valproic acid (Rendic, 2002). It also catalyzes the bioactivation of several carcinogens including aflatoxin B1 and nitrosamines (Rendic, 2002). Members of the *CYP2A* subfamily are inducible by diverse xenobiotics, such as phenobarbital, 3-methylcholanthrene, dexamethasone, and several hepatotoxins (Camus-Randon et al., 1996; Rendic, 2002). Hepatitis B and C virus infection, cirrhosis, parasitic infestation have been reported to cause overexpression of *CYP2A6* (Kirby et al., 1996; Satarug et al., 1996), yet the mechanisms of regulation of *CYP2A6* expression are unknown. The murine ortholog of *CYP2A6*, *Cyp2a5*, is induced by numerous agents and conditions that decrease the total amount of P450 enzymes in the liver, such as hepatotoxins (Camus-Randon et al., 1996), infestation by liver parasites (Montero et al., 1999), and in a mouse model of fulminant hepatitis (Chemin et al., 1996). This particular expression pattern suggests that *Cyp2a5* may become a major P450 isoform in damaged liver.

Post-transcriptional control has proven to be an essential mode of regulation of *Cyp2a5* expression. Post-transcriptional up-regulation of *Cyp2a5* is achieved by mRNA stabilization and is accompanied by an increase in *CYP2A5* mRNA poly(A⁺) tail length (Aida and Negishi, 1991; Geneste et al., 1996). Increased mRNA half-life is mediated by interaction of the RNA-binding protein heterogeneous nuclear ribonucleoprotein (hnRNP) A1 with a putative hairpin loop in the 3'-untranslated region (UTR) of *CYP2A5* transcript (Tilloy-Ellul et al., 1999; Raffalli-Mathieu et al., 2002; Glisovic et al., 2003a). hnRNPA1 probably plays a major role in the stabilization of *CYP2A5* mRNA occurring in response to toxic chemicals, for the maintenance of constant *CYP2A5* mRNA levels during conditions of impaired transcription (Glisovic et al., 2003b), and it may contribute to *CYP2A5* up-regulation in pathological conditions (Raffalli-Mathieu et al., 2002). Whether similar regulation mechanisms apply to the close human ortholog *CYP2A6* is unknown. Binding of a 43-kDa cytosolic protein to the 3'-UTR of *CYP2A6* mRNA has been reported (Gilmore et al., 2001). A potential regulatory func-

ABBREVIATIONS: P450, cytochrome P450; hnRNP, heterogeneous nuclear ribonucleoprotein; UTR, untranslated region; ActD, actinomycin D; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; RT, reverse transcription; PARP, poly ADP-ribose polymerase; PAGE, polyacrylamide gel electrophoresis.

tion of the protein was proposed from an observed correlation between CYP2A6 protein levels and the intensity of the CYP2A6 RNA-protein complex formed in liver extracts from four individuals. The protein identity and its role in CYP2A6 expression remain unknown. In this report, we have investigated in detail the interaction of proteins with the 3'-UTR of CYP2A6 mRNA. We detected five protein complexes binding to the 3'-UTR of CYP2A6 mRNA, one of which is hnRNP1. We identified a high-affinity hnRNP1 binding site within CYP2A6 3'-UTR and present a detailed analysis of the RNA-protein interaction. In addition, we show that the post-transcriptional regulation of *Cyp2a5* and *CYP2A6* expression share notable similarities. Our observations strongly suggest that hnRNP1 is a key regulator of the *CYP2A6* gene.

Materials and Methods

Isolation and Treatment of Primary Human Hepatocytes.

Human liver sample was obtained from a 79-year-old man who underwent right lobectomy for a metastasis of a colorectal tumor. Viral serologic analysis (hepatitis B virus, hepatitis C virus, HCV, and human immunodeficiency virus) was negative. The tissue encompassing the tumor was dissected by the surgeon in the surgery room and sent for pathologic studies, while the remaining encapsulated downstream tissue was used for hepatocyte preparation. No information on the patient was available in our laboratory, apart from the reason for surgical resection, age, and gender. Importantly, and as accepted by the French National Ethics Committee, pathological examination of the surgical specimen was in no way hindered by the procedure used to obtain primary hepatocytes; the tissue sample used for this purpose would otherwise have been immediately discarded. Hepatocytes were isolated and cultured as described previously (Pichard et al., 1992; Pichard-Garcia et al., 2002). The viability of cells before plating was determined using the trypan blue exclusion test and was 80%. Four million cells in 3 ml of culture medium were placed into 25-cm² flasks precoated with type I collagen. The serum-free culture medium (Pichard-Garcia et al., 2002) was changed every 48 to 72 h. Cultures were maintained at 37°C in a humid atmosphere of air and 5% carbon dioxide. In some experiments, cultures were treated for 24 h with 4 μ M ActD or carrier (dimethyl sulfoxide), and RNA or proteins extracts were prepared as described below.

Culture of HepG2 and HeLa Cells. HepG2 hepatocellular carcinoma cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were propagated in 10-cm plates (Corning, Palo Alto, CA) in minimal essential medium containing 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, minimal essential medium nonessential amino acids, and 1 \times antibiotic/antimycotic solution. HeLa cells were propagated in Dulbecco's modified Eagle's medium with 10% fetal calf serum. All cell culture products were purchased from Invitrogen (Carlsbad, CA). The cells were kept in an atmosphere of 5% CO₂ at 37°C in a humidified incubator and were subcultured at least two times per week.

Isolation of Cytoplasmic and Nuclear Proteins. Protein extracts were prepared as described previously (Glisovic et al., 2003b). In brief, primary human hepatocytes or HepG2 cells were scraped into PBS and centrifuged at 2000g for 30 s. The pellet was resuspended in buffer A (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, and 0.4% Igepal) and incubated on ice for 1 h. The cells were homogenized and centrifuged at 12,000g, 4°C, for 10 min. The supernatant (crude cytoplasmic fraction) was stored at -80°C. The nuclear pellet was resuspended in buffer B (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.4% Igepal) and homogenized. The nuclear extract was

centrifuged at 12,000g for 15 min at 4°C. The supernatant (nuclear proteins) was stored at -80°C. Protein concentrations were measured by the Lowry method (Lowry et al., 1951).

Preparation of Radiolabeled RNA Probes. Different segments of the CYP2A6 cDNA contained within the plasmid pCOH 17.1, generously donated by Hannu Raunio (University of Oulu, Finland), were amplified by using PCR (Fig. 1). Sense oligonucleotides contained the T7 RNA polymerase promoter 5'-TAATACGACTCATATAGGGAGA-3' at the 5' end. PCR-amplified products were transcribed with T7 RNA polymerase in the presence of ³²P-UTP (800 Ci/mmol; Pharmacia, Peapack, NJ) according to the manufacturer's instructions (Promega, Madison, WI). After digestion of the DNA template using RQ1 DNase, unincorporated nucleotides were removed from transcripts by the use of a microspin G-50 column (Amersham Biosciences AB, Uppsala, Sweden). All probes were checked for quality by electrophoresis through a denaturing polyacrylamide urea gel followed by autoradiography.

UV Cross-Linking. Binding reactions using nuclear or cytoplasmic extracts and the indicated RNA probes were performed as described by Geneste et al. (1996). The samples were irradiated for 20 min with UV light at an intensity of 5225 μ J/cm² in a Spectrolinker XL-1000 UV cross-linker (Spectronics, Westbury, NY). Free RNA was digested with 2 μ g of RNase A (Invitrogen, Täby, Sweden) at 37°C for 20 min. The samples were denatured under nonreducing conditions at 95°C for 10 min and separated on a 12% SDS-PAGE gel. Visualization was performed by autoradiography.

Competition Assays. A 258-nucleotide sequence corresponding to nucleotides 751 to 1009 (GenBank accession no. NM_00762) in the coding region of CYP2A6 cDNA was amplified via PCR using AmpliTaq Gold hot start *Taq* DNA polymerase and the plasmid pCOH17.1 containing CYP2A6 cDNA as a template. After a *Taq* activation step at 94°C for 1 min, amplification was performed as follows: 39°C, 15 s; 55°C, 30 s; and 72°C, 15 s, 40 times. Sequence of the primers were: sense, 5'-T7-ATAGCCAAGAAGGTGGAGCA-3'; and antisense, 5'-CTCTGTCAATCTCCTCATGGA-3'. The 234-nucleotide probe from the 3'-UTR of CYP2A6 was amplified as described previously. PCR amplified products were transcribed with T7 RNA polymerase in the presence of unlabeled ribonucleotides using the Ribomax kit (Promega) according to the manufacturer's instructions. After digestion of the DNA template with 2.5 U of RQ1 DNase, unincorporated nucleotides were removed from transcripts by the use of a microspin G-50 column (Amersham Biosciences AB), followed by phenol chlo-

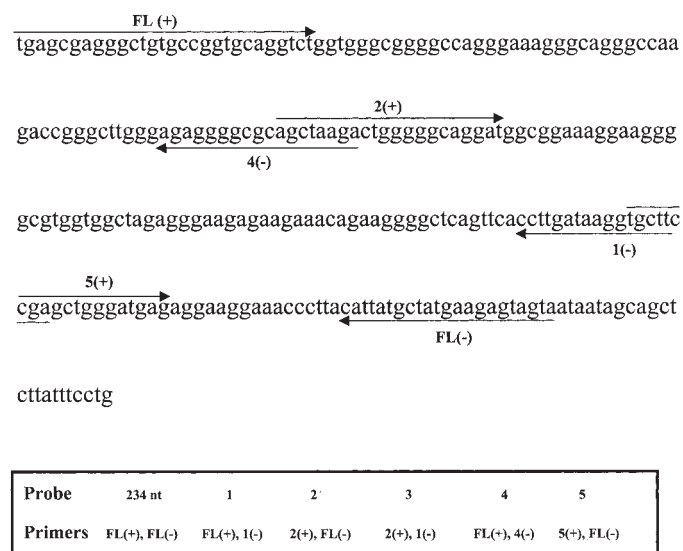


Fig. 1. Primers used to synthesize the different radioactive probes. The entire CYP2A6 3'-UTR is presented with primers indicated by arrows (+, sense; -, antisense). The combination of primers used for each probe is indicated.

roform extraction. Cold competitor RNA (1.7–177 ng; 10–1000 M excess) or 0.5 to 2.0 μ g of yeast tRNA in equal volumes were added to a standard UV cross-linking reaction containing 1 μ g of yeast tRNA before incubation with the 234-nucleotide 3'-UTR radioactive probe. Cross-linking was then performed as described above, and the reactions were separated on an SDS-PAGE gel. The gel was visualized by autoradiography after drying.

Partial Proteolysis of the RNA/Protein Complexes. Partial proteolysis of RNA/protein complexes was performed essentially as described by Hamilton et al. (1993). In brief, UV cross-linking reactions with human hepatocyte nuclear extracts were performed. Immediately after adding RNase A, 100 ng of trypsin (Roche Applied Science, Indianapolis, IN) was added to the samples. The samples were then separated by SDS-PAGE and visualized by autoradiography.

Immunoprecipitation of the RNA/Protein Complexes. Immunoprecipitation was performed essentially as described by Hamilton et al. (1993). In short, a UV cross-linking reaction with 15 μ g of human hepatocyte actinomycin D-treated cytoplasmic proteins or untreated HeLa nuclear proteins and the 234-nucleotide probe from CYP2A6 was performed. The UV cross-linked samples were added to 2 μ l of polyclonal hnRNP A1 antiserum, 2 μ l of pre-immune serum, or 2 μ l of monoclonal hnRNP I antibody (Zymed Laboratories, South San Francisco, CA), followed by immunoprecipitation with protein A-Sepharose beads (Amersham Biosciences AB). The samples were washed five times with 1 \times PBS, and the immunoprecipitated UV cross-linked complexes were denatured, separated on a 12% SDS-PAGE gel, and visualized by autoradiography.

RNA Isolation and Semiquantitative RT-PCR Analysis. Because of the limited material available from human donors, a strategy employing RT-PCR was chosen to measure RNA levels in primary human hepatocytes. Total cellular RNA was isolated from treated and untreated human primary hepatocytes using the RNeasy Mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. To ensure the lack of genomic DNA contamination, 30 μ g of total RNA was digested for 15 min at 37°C in a total reaction volume of 50 μ l using 2.5 U of RQ1 RNase-free DNase (Promega). The RNA was then purified by phenol chloroform extraction. Reverse transcription of 0.5 to 1 μ g of total RNA was performed using an oligo dT₁₆ primer and murine leukemia virus reverse transcriptase according to the manufacturer's instructions (PerkinElmer Life and Analytical Sciences, Boston, MA) in a total volume of 22 μ l.

Pre-RT annealing of oligo dT primer was performed for 10 min at 65°C, followed by addition of the murine leukemia virus reverse transcriptase. An RT reaction was then performed for 15 min at 42°C and 5 min at 99°C. All RT reactions were stored at –20°C or used immediately for duplex PCR using gene-specific CYP2A6 or CYP2B6 and β -actin control primers. The gene-specific primers for human CYP2A6, CYP2B6, and β -actin were of the same sequence as described previously by Lindell et al. (2003) and Strassburg et al. (1997). PCR was performed using AmpliTaq Gold hot start *Taq* DNA polymerase (Applied Biosystems, Foster City, CA) using 1.75 μ l of cDNA from the RT reaction as a template in a total reaction volume of 50 μ l. After an initial *Taq* activation step of 3 min at 95°C, the CYP2A6 or CYP2B6 gene-specific primers were added to the reaction. PCR was performed as follows: 2 min at 94°C, 1 min at 50°C, and 2 min at 72°C for eight (CYP2A6) or 16 (CYP2B6) cycles, followed by the addition of the β -actin control primers. The above PCR program was then run an additional 17 (CYP2A6) or 15 (CYP2B6) cycles. The β -actin, CYP2A6, and CYP2B6 amplified fragments are 202, 420, and 551 bp long, respectively. Extensive optimization was carried out to ensure linearity of the CYP2A6, CYP2B6, and β -actin signal response, after which RT-PCR semiquantitative analysis was performed. All RT and PCR reactions were repeated at least three times to ensure reproducibility, and identical results were obtained.

The relative band intensities for CYP2A6, CYP2B6, and β -actin were quantitated using NIH Image software version 1.62f ([\[rsb.info.nih.gov/ni-image/\]\(http://rsb.info.nih.gov/ni-image/\)\). CYP2A6 and CYP2B6 expression were normalized using the \$\beta\$ -actin signal before plotting.](http://</p>
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Western Blot Assay. The denatured samples were run on a 4% stacking and 12% SDS-PAGE separating gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Hybond ECL; Amersham Biosciences AB). The membrane was blocked in 5% milk and 0.1% Tris-buffered saline-Tween 20. The primary antibodies used were as follows: ACT-1 polyclonal anti-hnRNP A1 (Hamilton et al., 1997), 1:5000; monoclonal anti-poly ADP-ribose polymerase (PARP), 1:500, to access nuclear loading and apoptotic state of the cells; or polyclonal anti-actin (Santa Cruz Biochemicals, Santa Cruz, CA), 1:250, to assess cytoplasmic protein loading. Detection was performed using ECL reagents according to the manufacturer's instructions (Amersham Biosciences AB). Densitometric analysis was performed using National Institutes of Health image software version 1.62f, and the hnRNP A1 signal was normalized to actin in cytoplasmic extracts and PARP in nuclear extracts.

Conformational Analysis of the Secondary Structure of the CYP2A6 3'-UTR. Secondary structure analysis was performed using mfold version 3.1 (Mathews et al., 1999; Zuker et al., 1999) using the web-based server at Rensselaer Polytechnic Institute (<http://www.bioinfo.rpi.edu/applications/mfold>). Only the most stable structure is shown.

Alignment of the 3'-UTR from CYP2A5 and CYP2A6. Alignment of the primary nucleotide sequence from the 3'-UTR of CYP2A5 and CYP2A6 was performed using the Geneworks version 2.5 program (Oxford Biomedical Research, Oxford, MI). The GenBank 3'-UTR accession numbers for CYP2A6 cDNA and *Cyp2a5* cDNA were NM_00762 and BC046605, respectively. Identical nucleotides are marked as shaded boxes.

Statistical Analysis. Statistical analysis of scanned RT-PCR data were performed using Minitab statistical software version 13.32 (Minitab Inc., State College, PA). Data were processed using a two-sample (unpaired) Student's *t* test at a 95% significance level. Data are plotted as means \pm S.D. Asterisk indicates significance level of *p* < 0.05.

Results

The Human CYP2A6 and Mouse CYP2A5 3'-UTR Show Highly Homologous AG-Rich Blocks. The 3'-UTR of the mouse CYP2A5 mRNA contains *cis*-acting sequences essential for the post-transcriptional regulation (Glisovic et al., 2003a). To study the possibility that this mode of regulation is evolutionarily conserved in humans, a comparison of the mouse CYP2A5 mRNA and the human CYP2A6 mRNA using Geneworks version 2.5.1 software (Fig. 2A) was performed. This alignment showed a 41% nucleotide identity between the two 3'-UTR sequences, reflecting the divergence of these two orthologs across species. However, two purine-rich blocks, 15 and 12 nucleotides long, which we have named AG-rich blocks I and II, are strikingly homologous between CYP2A5 and CYP2A6 (Fig. 2A). Both of these regions contain 5'-G/AGAAGA/G-3' sequences highly reminiscent of known hnRNP A1 binding sites (Fig. 2B; Tilloy-Ellul et al., 1999; Huang and Lai, 2001; Damgaard et al., 2002; Pollard et al., 2002; Guil et al., 2003; Rooke et al., 2003). Block I contains two repeats of this sequence, whereas block II contains one. An additional repeat is found upstream of block I in CYP2A6.

Several Proteins from Human Hepatocytes Interact with CYP2A6 3'-UTR. To identify proteins interacting with the 3'-UTR of CYP2A6 mRNA, UV cross-linking experiments were performed using protein extracts from human primary hepatocytes. Nuclear and cytoplasmic extracts were incubated with a radiolabeled RNA probe spanning 234 nucleo-

The 34-/37-, 54-, 77-, and 135-kDa Protein Complexes Are Specific for CYP2A6 3'-UTR. To analyze the specificity of the RNA/protein complexes, UV cross-linking reactions with primary human hepatocyte nuclear extracts and the radiolabeled CYP2A6 3'-UTR 234-nucleotide probe were per-

Fig. 2. Analysis of the CYP2A6 3'-UTR sequence. A, alignment of the 3'-UTR cDNA sequence of *Cyp2a5* and *CYP2A6*. The alignment was performed using Geneworks software. Hyphens indicate gaps in the alignment. Identical nucleotides are marked using a gray box. The AG-rich blocks I and II in the *CYP2A6* sequence are indicated by a solid line, and the *CYP2A5* 71-nucleotide *cis*-element described by Tilloy-Ellul et al. (1999) and Geneste et al. (1996) is delineated by a dotted line. B, comparison of known hRNPA1 binding sequences.

RNA	Sequence (5'→3')	Reference
CYP2A6 AG rich region I	<u>AGAAGAAACAGAAGG</u>	This Work
Cyp2A5 (right side of hairpin loop)	UAGCCAA <u>AGAAG</u> GGGAAA	Geneste et al., 1996
Mouse hepatitis virus 3'UTR mRNA	GAGAG <u>AAG</u> UU	Huang et al., 2001
C-src exon N 1	UUAGGAG <u>GAAGG</u> UGCCAUCGAUGUCAG	Rooke et al., 2003
HIV-1 TAT pre-mRNA	AAGGAAU <u>AGAAGAAAG</u>	Damgaard et al., 2002
Gαs exon 3	GCGAAGAGGACCCGCAGGCTGCA <u>AGGAGCAA</u>	Pollard et al., 2002
c-H-Ras ISS1	GGCAGUG <u>AGGGAGGCG</u> AGGG	Guil et al., 2003

formed. Yeast tRNA, unlabeled 234-nucleotide 3'-UTR probe, or 258 nucleotides of CYP2A6 coding region were used as unlabeled competitors in the binding reactions (Fig. 5).

CYP2A6 3'-UTR competed much more readily for the 34-/37-, 54-, 77-, and 135-kDa complexes than tRNA or CYP2A6 coding region, suggesting that these protein complexes are 3'-UTR specific. The 25-kDa complex seemed to bind CYP2A6 3'-UTR less specifically because it was as efficiently competed out by the 3'-UTR and coding region of CYP2A6 mRNA.

Identification of the 34-/37-kDa Protein in Human Primary Hepatocytes. To investigate the possibility that the human 34-/37-kDa protein binding to the 234-nucleotide probe from CYP2A6 was hnRNP A1, UV cross-linked protein complexes bound to the 234-nucleotide probe were subjected to partial proteolysis in conditions known to allow the cleavage of hnRNP A1 into a smaller, approximately 25-kDa protein known as the UP1 fragment (Hamilton et al., 1993),

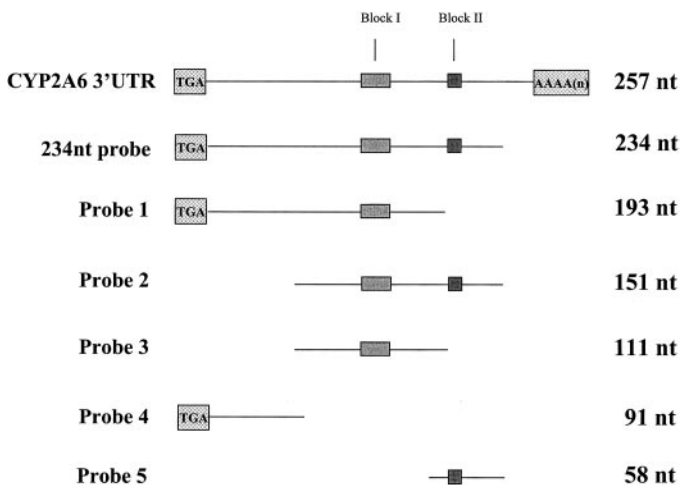


Fig. 3. Schematic of the radiolabeled RNA probes from the CYP2A6 3'-UTR used in this study. The AG-rich homology regions between CYP2A5 and CYP2A6 mRNA are highlighted (gray boxes).

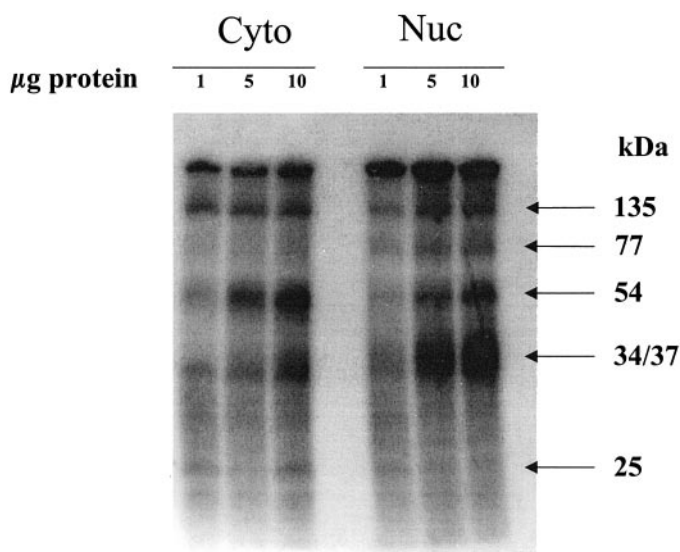


Fig. 4. Detection of proteins binding to the 3'-UTR of CYP2A6. UV cross-linking was performed using the radiolabeled 234-nucleotide probe (full-length 3'-UTR), and various amounts of cytoplasmic and nuclear extracts from primary human hepatocytes as indicated. Molecular masses of the RNA/protein complexes are shown.

which retains RNA binding activity. Trypsin digestion caused a reduction of the 34-/37-kDa complex and an increase of a 25-kDa complex (Fig. 6). The results are consistent with the idea that the 34-/37-kDa protein bound to the 3'-UTR of CYP2A6 is hnRNP A1.

Immunoprecipitation of the 34-/37-kDa Protein Complex Identifies It as hnRNP A1. To confirm the identity of the 34-/37-kDa protein found in human hepatocyte nuclear extracts, immunoprecipitation with an antibody specific for hnRNP A1 was performed. The UV cross-linked complexes were immunoprecipitated with anti-hnRNP A1 antiserum, pre-immune serum, or a monoclonal antibody against hnRNP I (polypyrimidine tract binding protein). We observed repeatedly that only the hnRNP A1 antiserum resulted in a positive signal (Fig. 7), showing that the 34-/37-kDa protein bound to the 3'-UTR of CYP2A6 is hnRNP A1 or a close relative.

A positive signal corresponding to the 77-kDa complex but not the 54- or 135-kDa complexes was also observed, suggesting that the 77-kDa complex binding to the 3'-UTR of CYP2A6 mRNA is composed of a dimer of the hnRNP A1 protein.

Mapping of the Minimal Binding Site for hnRNP A1 to the 3'-UTR of CYP2A6. To locate the binding region(s) of the hnRNP A1 protein and the other unknown RNA binding proteins to the CYP2A6 3'-UTR, the 234-nucleotide probe was truncated to different sizes (Fig. 3). Because the purine-rich blocks I or II may be responsible for hnRNP A1 binding activity, the truncations were designed to eliminate one or both of these blocks. These probes were then UV cross-linked to nuclear extracts from untreated human hepatocytes, and the resulting cross-linked complexes were separated by SDS-PAGE (Fig. 8). The shortest probe to maintain significant binding activity of hnRNP A1 in nuclear extracts was probe 3, which contains block I. In contrast, the 3' end or the 5' end alone of the 234-nucleotide probe seemed to have little positive contribution to the primary binding activity of hnRNP A1 as evidenced by lack of significant hnRNP A1 binding to

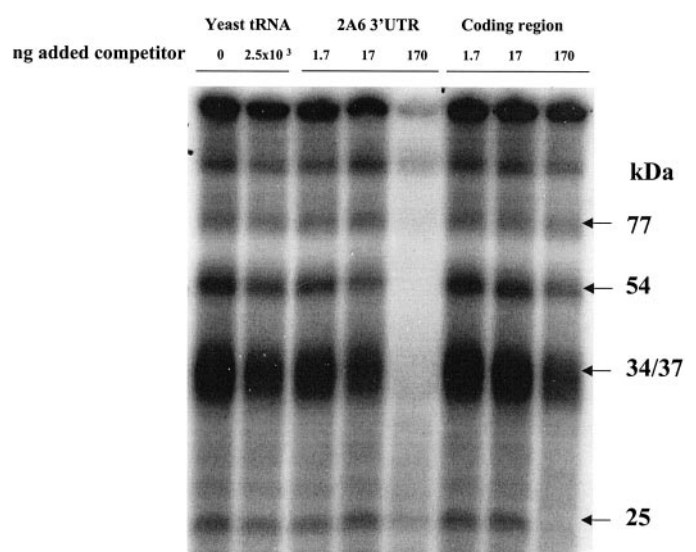


Fig. 5. Specificity of proteins binding to the 3'-UTR of CYP2A6. UV cross-linking was performed using the radiolabeled 234-nucleotide probe and 5 μ g of nuclear extracts from primary human hepatocytes in the presence of various amounts of unlabeled competitor as indicated. The locations of the RNA/protein complexes are indicated.

probes 4 and 5. This is in accordance with the observation that probes 1 and 2 showed no increase in complex formation compared with probe 3. All truncated probes showed decreased binding activity of hnRNPA1 compared with the 234-nucleotide probe, suggesting that the presence of both the 3' and the 5' end together with the primary binding region are necessary for optimal hnRNPA1 binding.

The smallest probe to maintain significant binding activity of the nuclear 77-kDa protein was probe 3, suggesting that the primary binding site of this protein is within probe 3, whereas the shortest probe to maintain binding activity of the 135-kDa nuclear protein was probe 4. The mapping of the primary binding activity of both the 34-/37- and 77-kDa nuclear complexes to the same region of the 3'-UTR supports

the idea that the 77-kDa complex may, in fact, be a dimer of the hnRNPA1 protein.

Binding of hnRNPA1 from Hepatocellular Carcinoma Cells to the 3'-UTR of CYP2A6. To investigate protein binding activity toward the 3'-UTR of CYP2A6 in human primary hepatocytes compared with a human hepatoma cell line, nuclear extracts from untreated HepG2 cells were UV cross-linked to the 3'-UTR of CYP2A6. The highest binding activity of all probes was seen with probe 2, whereas the primary binding site of the 34-/37-kDa protein (Fig. 9) was contained within probe 3. The primary binding site of hnRNPA1 from HepG2 and human hepatocyte nuclear extracts to the 3'-UTR of CYP2A6 maps thus to the same minimal binding region. However, unlike in human primary hepatocytes, probe 2 supports most efficient binding of the hnRNPA1 protein in the cancer cell line, suggesting that the binding characteristics of hnRNPA1 in the hepatoma cell line are different from those observed in primary human hepatocytes.

Predicted Secondary Structures of the CYP2A6 3'-UTR Probes. To investigate the possible influence the secondary structure of the 3'-UTR of CYP2A6 may have on hnRNPA1 binding, RNA secondary structure predictions of the 234 nucleotides and probes 1 to 5 were made. A comparison of the predicted structures (Fig. 10) shows that the block I and block II homologies have a consistent tendency to be part of open, single-stranded loops in all predicted RNA secondary structures. These loops are rich in 5'AGAAGA/G 3' repeats, which show high similarity to the 5'AGAAG 3' high-affinity binding site for hnRNPA1 in mouse hepatitis virus RNA identified by Huang et al. (Huang and Lai, 2001) in both primary sequence and secondary structure.

Effect of ActD Treatment on the 34-/37- and 54-kDa Proteins Binding Activity. Interruption of transcription in mouse hepatocytes and exposure of mice to liver toxins results in dramatic changes to hnRNPA1 subcellular localization and binding activity to CYP2A5 mRNA (Glisovic et al., 2003b). This has been shown to affect *Cyp2a5* expression by increasing the stability of CYP2A5 mRNA (Glisovic et al.,

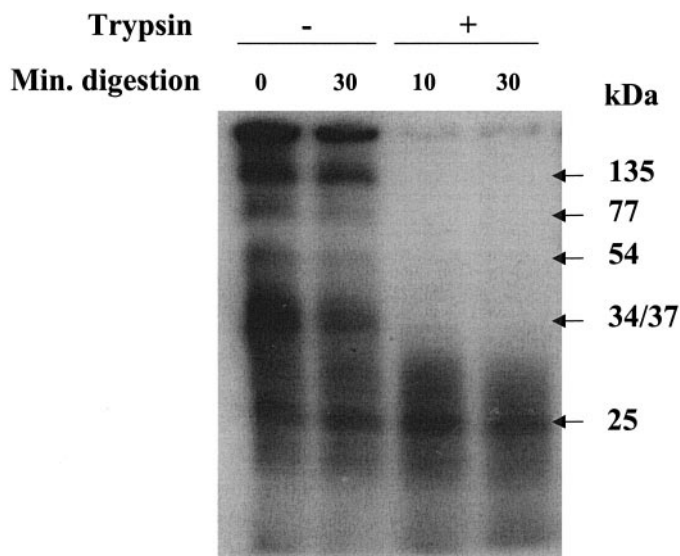


Fig. 6. Partial proteolysis of the RNA/protein complexes bound to the 3'-UTR of CYP2A6. Trypsin was added to a UV cross-linking reaction containing the radiolabeled 234-nucleotide probe and nuclear extracts from untreated primary human hepatocytes for the indicated times. The locations of the complexes are shown.

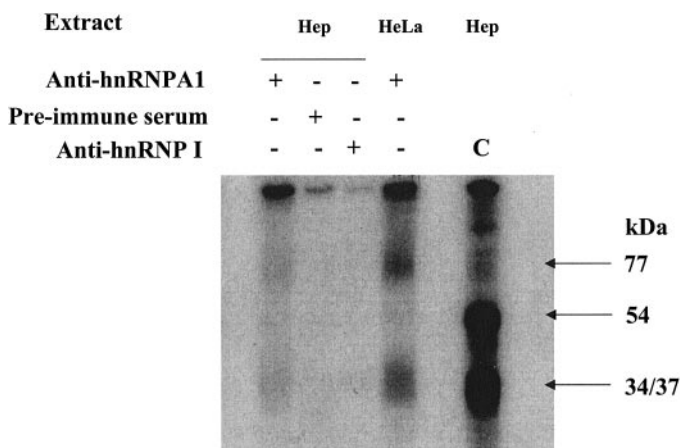


Fig. 7. Immunoprecipitation of hnRNPA1 bound to the 3'-UTR of CYP2A6. UV cross-linking was performed with human hepatocyte (Hep) nuclear extracts or HeLa cell nuclear extracts (5 μ g of proteins) and the 234-nucleotide probe. Antibodies were added to the reaction mixture as indicated, and immunoprecipitation was performed as described under *Materials and Methods*. Molecular masses of the complexes are indicated. "C" denotes the control sample where UV cross-linking has been performed without immunoprecipitation.

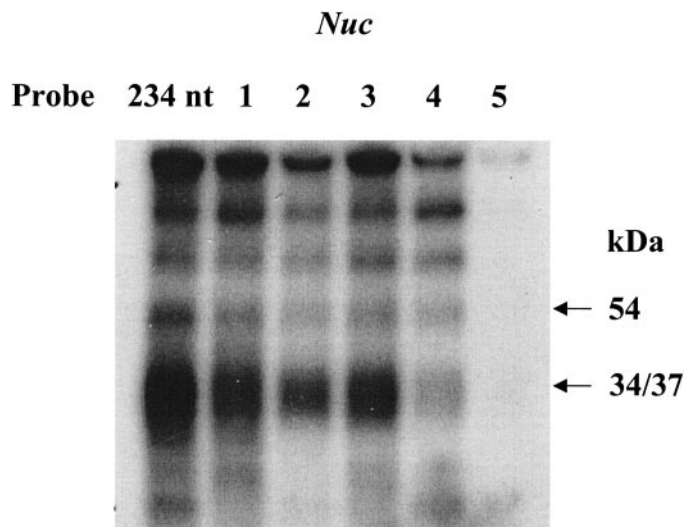


Fig. 8. Mapping of the primary binding site of hnRNPA1. The probes described in Fig. 3 were used in UV cross-linking experiments with 5 μ g of nuclear extract from primary human hepatocytes. The 34-/37-, 54-, and 77-kDa complexes are shown.

2003b). It is, however, unknown whether the human ortholog of this gene, *CYP2A6*, shows a similar mode of regulation in response to cellular stress. To test whether interruption of transcription in human hepatocytes would result in similar changes in hnRNP A1 protein binding to the 3'-UTR of *CYP2A6*, nuclear or cytoplasmic protein extracts from untreated or ActD-treated cells were UV cross-linked to the *CYP2A6* 234-nucleotide probe. Cross-linking analysis (Fig. 11A) revealed a striking decrease of the hnRNP A1 binding activity in the nucleus of ActD-treated cells and a corresponding increase in cytoplasmic hnRNP A1 protein binding activity. A similar modification of the 54-kDa protein subcellular localization in response to ActD is observed. Western

blot analysis (Fig. 11, B and C) of the protein extracts revealed a sharp decrease in the nuclear levels of hnRNP A1 after 24 h of treatment with 4 μ M ActD, whereas cytoplasmic levels remained unchanged.

CYP2A6 mRNA Is Stabilized by Interruption of Transcription with ActD. *CYP2A5* mRNA is stabilized in response to inhibition of cellular transcription (Glisovic et al., 2003b). To determine whether the level of *CYP2A6* mRNA is similarly regulated, primary human hepatocytes were treated for 24 h with 4 μ M ActD, a concentration of ActD that completely inhibits RNA polymerase II-dependent transcription (Perry and Kelley, 1970). The levels of *CYP2A6* mRNA in control and ActD-treated cells were measured using semi-quantitative duplex RT-PCR using the levels of β -actin mRNA as a reference. As a control, the level of *CYP2B6* mRNA, a P450 isoform known to be regulated primarily at the transcriptional level (Pascucci et al., 2003), was measured.

The results from the RT-PCR experiments (Fig. 12, A and B) show, after 24 h, significantly ($p < 0.03$, $n = 3$) decreased levels of *CYP2A6* mRNA in control cells but unchanged levels of *CYP2A6* mRNA in ActD-treated cells. *CYP2A6* mRNA levels were calculated to be approximately 2.3 fold higher in ActD-treated cells compared with controls. In contrast, *CYP2B6* mRNA levels were not significantly changed by ActD treatment. β -Actin mRNA levels were not affected in either treated or untreated cells.

Discussion

The human xenobiotic-metabolizing enzyme *CYP2A6* has been shown to be overexpressed in cases of cirrhosis and viral hepatitis, supporting the view that it is induced in damaged liver, in a similar manner as its murine ortholog, *Cyp2a5*. In the present study, we have addressed molecular mechanisms

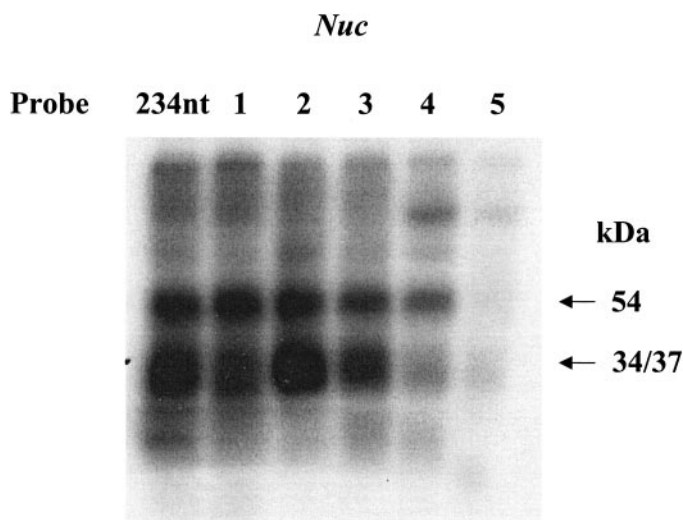


Fig. 9. Binding of hnRNP A1 from HepG2 cells. The probes indicated in Fig. 3 were used in UV cross-linking experiments with 5 μ g of nuclear extract from untreated HepG2 cells. The locations of the complexes are shown.

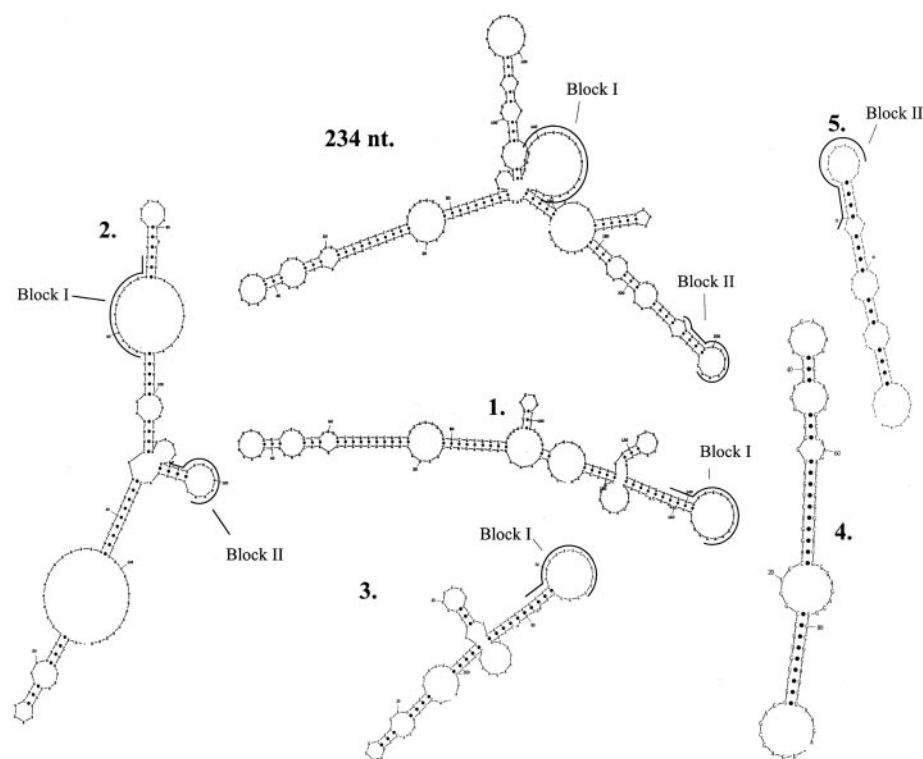


Fig. 10. Predicted secondary structures of the probes used in this study (234 nucleotides, 1-5, see Fig. 3). Only the most stable structure is shown. The locations of the AG-rich homology blocks I and II (when present) are indicated.

underlying the regulation of the expression of the *CYP2A6* gene. We found that *CYP2A6* expression can be affected post-transcriptionally, and the post-transcriptional regulation seems to involve interaction of the hnRNP A1 protein with *CYP2A6* mRNA. We show that *CYP2A6* mRNA is stabilized when transcription is impaired.

Post-transcriptional regulation is a major mode of control of expression of *Cyp2a5*, and it involves the interaction of the RNA-binding protein hnRNP A1 with a specific RNA motif in the 3'-UTR of the *CYP2A5* transcript (Raffalli-Mathieu et al., 2002; Glisovic et al., 2003a). Therefore, we searched for sequence homologies between the 3'-UTRs of *CYP2A5* and *CYP2A6*. Although only 41% nucleotide identity is observed, two 12- to 14-nucleotide-long AG-rich motifs (blocks I and II) are very well conserved between mouse and humans. According to the sequence alignment, block II lies within a region corresponding to the primary binding site of hnRNP A1 in *CYP2A5* mRNA, and block I is located eight nucleotides upstream of this region. Moreover, the sequences G/AGAAGA/G contained in the AG-rich blocks show high similarity to the 5'AGAAG 3' high-affinity hnRNP A1 binding site found in the 3'-UTR of mouse hepatitis virus RNA (Huang and Lai, 2001) and to the purine-rich 3' splice site of *c-src* mRNA exon N1 composed of the sequence 5'GAGGAAG-

GUG 3' recently described by Rooke et al. (2003) to bind hnRNP A1. The importance of AG-rich motifs for high-affinity hnRNP A1 binding has also been reported for *c-H-ras* and HIV1 TAT pre-mRNAs (Damgaard et al., 2002; Guil et al., 2003). Altogether, these observations suggest that RNA-protein interactions take place at the 3'-UTR of *CYP2A6* and probably involve hnRNP A1, as in the case of *CYP2A5*.

Our RNA-binding experiments demonstrate that several protein complexes present in human hepatocytes interact specifically with the 3'-UTR of *CYP2A6*. The RNA-protein complex of highest intensity (detected at 34/37 kDa) contains hnRNP A1 or a closely related protein with identical size, binding specificity, subcellular localization, partial proteolysis pattern, and immunological properties. Immunological evidence and mapping data support the idea that the 77-kDa nuclear complex is a dimer of the 34-/37-kDa hnRNP A1 protein, either with itself or with another protein of similar size. The identity of the proteins in the other complexes was not investigated in this study.

Our results are not in accordance with those of Gilmore et al. (2001), who found one protein of 43 kDa binding to *CYP2A6* 3'-UTR. The distinct sizes might arise from different experimental conditions. However, the 43-kDa protein is described as a cytosolic protein, which argues against the idea that the 43-kDa and 34-/37-kDa proteins may, in fact, be the same protein. One important difference between the two studies is that Gilmore et al. used tissue homogenates containing proteins from different cell types, whereas we have

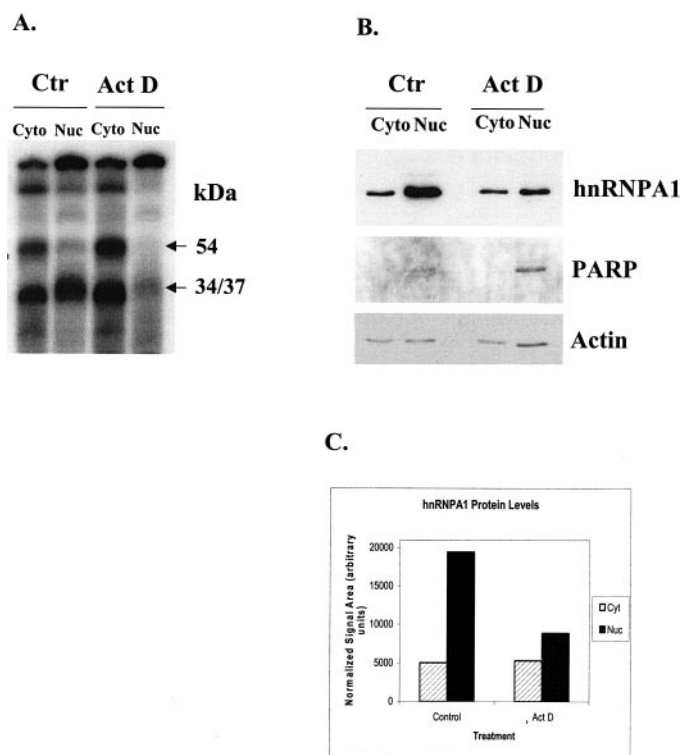


Fig. 11. The 34-/37-kDa binding activity in primary human hepatocytes is transferred from the nucleus to the cytoplasm upon ActD treatment. A, UV cross-linking using the radiolabeled 234-nucleotide probe and 6 μ g of cytoplasmic and 4 μ g of nuclear extracts from untreated or treated (4 μ M ActD, 24 h) cells. B, Western blot. 25 μ g of control or ActD-treated nuclear or cytoplasmic extracts were separated using SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blotted using anti-hnRNP A1 rabbit polyclonal (ACT-1) antibody, then stripped and reblotted using anti-PARP mouse monoclonal or anti-actin goat polyclonal antibody (Santa Cruz Biochemicals) to access loading. C, densitometric analysis of the Western blot in B. The signal for hnRNP A1 was corrected for differences in loading using the actin signal in the cytoplasm and PARP signal in nuclear extracts.

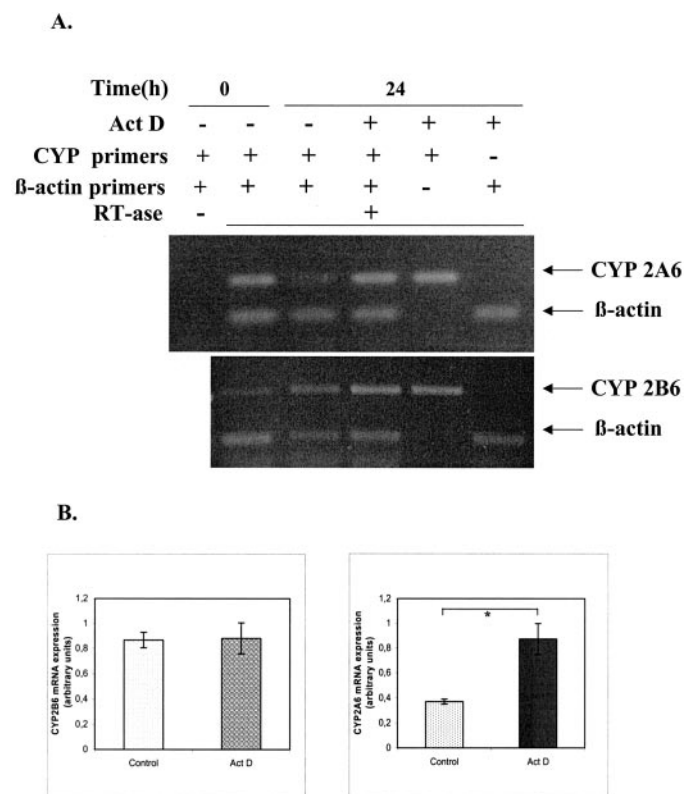


Fig. 12. CYP2A6 mRNA is stabilized in response to interruption of transcription with ActD. Primary human hepatocytes were treated for 24 h with 4 μ M ActD or carrier (dimethyl sulfoxide) only, and RNA was prepared. A, semiquantitative duplex RT-PCR performed on RNA from treated and carrier only treated cells. B, densitometric analysis of CYP2A6 and CYP2B6 mRNA expression normalized to β -actin signal in ActD-treated and carrier-treated cells. $n = 3$, $p < 0.03$.

used protein extracts from primary hepatocytes in culture, which may explain why distinct binding patterns are found.

Detailed mapping of hnRNP A1 binding site within CYP2A6 3'-UTR reveals that the primary binding site of hnRNP A1 to CYP2A6 3'-UTR lies within the 111 central nucleotides of the 3'-UTR, containing AG-rich block I. The strongest binding is observed only when the 3'-UTR is complete, indicating that sequences flanking the primary binding region cooperate to provide the optimal environment for hnRNP A1 binding. This suggests that the 3'-UTR's secondary structure plays an important role for the binding. Analysis of the putative secondary structures of the different probes showed consistently that blocks I and II are at least partially single stranded as part of loops, raising the possibility that these blocks may be single stranded *in vivo* and that these sequences may form secondary structures allowing hnRNP A1 to bind with high affinity. This is reminiscent of CYP2A5 mRNA, where the hnRNP A1 primary binding site is found at the tip of a putative hairpin-loop (Tilloy-Ellul et al., 1999).

We sought to study the binding pattern of hnRNP A1 present in a human liver cancer cell line, HepG2, to CYP2A6 3'-UTR, because altered expression of this gene has been associated with the presence of chronic inflammation in hepatocellular carcinoma (Raunio et al., 1998). hnRNP A1 from HepG2 cancer cells and primary hepatocytes exhibited different binding characteristics to CYP2A6 3'-UTR. In particular, sequences 3' of the primary binding site promoted the binding of hnRNP A1 present in HepG2 cells, whereas the opposite was observed with protein extracts from primary hepatocytes. This may arise from the presence of cell type-specific proteins affecting the binding of hnRNP A1 to CYP2A6 mRNA. It is also possible that post-translational modifications, such as phosphorylation and methylation, of hnRNP A1 are different in primary and cancer cells. In support of this, arginine methylation, known to affect the RNA binding activity of hnRNP A1 (Kim et al., 1998), is altered in HepG2 and other cancer cells, such as the human cervical cancer cell line HeLa (Gu et al., 1999). Interestingly, the binding pattern of hnRNP A1 to CYP2A6 mRNA was identical in HeLa and in HepG2 cells (data not shown). Although the functional significance of the distinct binding patterns remains to be studied, modified binding of hnRNP A1 to CYP2A6 mRNA in cancer cells is of particular interest, with respect to the increased CYP2A6 expression observed in certain liver tumors (Raunio et al., 1998).

Because our data revealed very similar RNA-protein interactions taking place at the 3'-UTR of CYP2A5 (Tilloy-Ellul et al., 1999) and CYP2A6 (this study), we hypothesized that CYP2A6 mRNA may be stabilized in response to inhibition of RNA polymerase II-dependent transcription in an analogous manner to *Cyp2a5* (Glisovic et al., 2003b) and several other genes, such as GM-CSF and transferrin receptor (Seiser et al., 1995; Esnault et al., 1998). This regulatory mechanism may reflect a means for the cells to maintain the expression of genes necessary for cell survival in certain stress conditions involving impaired transcription. We found that the level of CYP2A6 mRNA decreases with time as expected in nontreated cells (Meunier et al., 2000; Renwick et al., 2000) but remains high in the presence of a concentration of ActD preventing *de novo* pre-mRNAs synthesis, revealing stabilization of CYP2A6 mRNA by ActD. Moreover, our data show

that in human primary hepatocytes, ActD dramatically alters the subcellular distribution of hnRNP A1 and of the RNA-binding activity related to it. Transcription-dependent translocation of hnRNP A1 from the nucleus to the cytoplasm was first described in HeLa cells (Pinol-Roma and Dreyfuss, 1992), and we have previously shown that it also takes place in mouse primary hepatocytes (Glisovic et al., 2003b). Here, we observe only a marginal increase of cytoplasmic hnRNP A1 after exposure of human primary hepatocytes to ActD for 24 h. This may be explained by the long time frame of the treatment, during which the total cellular hnRNP A1 protein level probably starts to decrease. The large increase in CYP2A6 mRNA-hnRNP A1 cytoplasmic complex formation observed in these conditions probably resulted from a stimulation of the protein's RNA-binding activity, as has been observed in human T lymphocytes by Hamilton et al. (1997).

In conclusion, the present data and our earlier work (Geneste et al., 1996; Glisovic et al., 2003a, 2003b; Raffalli-Mathieu et al., 2002) suggest that hnRNP A1 is a regulator of expression of the human CYP2A6 gene, particularly in conditions of cellular stress. Studies aiming at understanding how hnRNP A1 may affect the stability of CYP2A6 mRNA and of how stress conditions affect the activity of hnRNP A1 are currently ongoing in our laboratory.

The CYP2A6 gene has been shown to be polymorphic, with large differences in enzyme activities between individuals (Raunio et al., 2001). The polymorphisms described thus far have been found in the mRNA coding region (Raunio et al., 2001), but it can be speculated that mutations affecting hnRNP A1 binding to CYP2A6 3'-UTR may alter CYP2A6 mRNA stability and thereby CYP2A6 expression. Interestingly, the expression of several human genes, such as the α -globin and cyclin D1 genes, has been shown to be disturbed by mutations affecting 3'-UTR-specific RNA-protein interactions (Conne et al., 2000). Mutations in the 3'-UTR of CYP2A6, and possibly other P450 genes, may contribute to a largely overlooked, novel source of genetic polymorphisms that can potentially affect drug and other xenobiotic metabolism.

Acknowledgments

We thank Dr. Hannu Raunio for the plasmid containing CYP2A6 cDNA, Monica Lindell for the use of her β -actin and CYP2A6- and CYP2B6-specific primers, and Angela Lannerbro for her expert technical assistance.

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