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Biochemical Pharmacology

Biochemical Pharmacology 62 (2001) 669–678
Molecular and cellular pharmacology

Identification of a 43-kDa protein in human liver cytosol that binds to the 3′-untranslated region of CYP2A6 mRNA

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Received 30 March 2000; accepted 4 December 2000

Abstract

Hepatic expression of cytochrome P450 2A6 (CYP2A6) varies widely in humans and is induced during hepatitis; however, the mechanism regulating CYP2A6 has not been established. The murine orthologue Cyp2a5 is regulated post-transcriptionally by mRNA stabilization. A 43-kDa protein that binds to the 3′-untranslated region (3′-UTR) of Cyp2a5 mRNA has been identified, but its role in mRNA stabilization is unclear. We hypothesized that similar interactions occur between cytosolic proteins in human liver and CYP2A6 3′-UTR mRNA. We identified, by RNA electrophoretic mobility shift assay, an hepatic cytosolic protein that binds specifically to sequences in the 3′-UTR of CYP2A6. Complexes did not form with denatured proteins and were eliminated with proteinase K digestion. Complex formation was inhibited with a molar excess of unlabeled CYP2A6 RNA but not by non-specific competitor RNA. Protein–mRNA interactions were not affected by probe denaturation, suggesting that RNA secondary structure is not essential for binding. UV cross-linking of complexes revealed RNA-binding proteins in both human and mouse liver cytosols with molecular masses of approximately 43 kDa. Using truncated RNA probes corresponding to various lengths of CYP2A6 mRNA, the protein-binding site was localized to a 50-nucleotide region between bases 1478 and 1527 of the 3′-UTR. Complex formation with hepatic cytosolic protein from four human subjects correlated with levels of hepatic CYP2A6 microsomal protein, suggesting a possible regulatory role. Further characterization of the RNA-binding protein, the primary binding site, and the influence of this interaction on CYP2A6 mRNA stability will help to elucidate the relevance of these findings to the post-transcriptional control of CYP2A6.

Keywords: Cytochrome P450; RNA binding protein; 3′-Untranslated region

1. Introduction

In humans, cytochrome P450 2A6 is involved in the metabolic activation of various chemical carcinogens including nitrosamines and aflatoxins [1–5]. While CYP2A6 expression varies greatly among individuals [6,7], the mechanism regulating CYP2A6 gene expression in humans is unknown. Regulation of Cyp2a5, the mouse orthologue that has high sequence complementarity and shares catalytic properties with CYP2A6 [8], has been studied extensively [9]. Cyp2a5 induction is associated with liver injury caused by a number of structurally diverse hepatotoxins including pyrazole [10], carbon tetrachloride [11], benzene [12], and various metal ions such as cobalt [13], cerium [14], and indium [15]. Moreover, induction of Cyp2a5 has been demonstrated in various rodent models of chronic liver injury by infectious agents. For example, Cyp2a5 is induced in HBV transgenic mice [16], in hamsters with liver fluke infestation [17], and in mice with hepatitis due to *Helicobacter hepaticus* [18]. There are also reports of overexpression of CYP2A6 in association with hepatitis and cirrhosis due to HBV or HCV infection [19,20]. There is accumulating evidence that post-transcriptional mechanisms are involved in the regulation of Cyp2a5. Nuclear run-off analysis has revealed that selective induction of Cyp2a5 by pyrazole

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Abbreviations: 3′-UTR, 3′-untranslated region; CYP, cytochrome P450; EMSA, electrophoretic mobility shift assay; HBV, hepatitis B virus; HCV, hepatitis C virus; hnRNP, heterogenous nuclear ribonucleoprotein; pBS, pBluescript; PCR, polymerase chain reaction; and TBE, Tris-borate-EDTA.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00720-1

is mediated post-transcriptionally via stabilization of Cyp2a5 mRNA [21,22]. To date, however, no studies have focused on the molecular mechanisms that regulate CYP2A6 expression.

The modulation of mRNA stability is an important mechanism of gene regulation, particularly for gene products that must be regulated within a narrow timeframe (e.g. immediate early genes) [23]. Inducible molecules (e.g. cytokines, oncogenes, transcriptional activators, enzymes) that are responsive to environmental, hormonal, or nutritional stimuli often have unstable mRNA [24–26]. There are numerous reports that RNA–protein interactions are critical in mediating post-transcriptional control of gene expression, and *trans*-acting protein factors that modulate mRNA stability are becoming increasingly characterized [27]. A number of *cis*-acting elements have been implicated in the regulation of mRNA stability by binding cytosolic proteins. Defined regions known as adenosine/uridine-rich elements in the 3′-UTR of many short-lived mRNAs appear to regulate gene expression. Among the best characterized is the iron responsive element involved in the reciprocal changes in the translation and mRNA stability of ferritin and transferrin receptor mRNA [28]. Recent evidence suggests that the post-transcriptional regulation of Cyp2a5 expression involves protein–mRNA interactions [29]. Several cytosolic proteins that bind specifically to the 3′-UTR of Cyp2a5 mRNA have been identified recently, suggesting a possible role in regulating Cyp2a5 message stability; however, this has not been established [29–31]. The objective of the present study was to determine whether similar interactions occur between human hepatic cytosolic proteins and the 3′-UTR of CYP2A6 mRNA. Using RNA EMSAs, we have identified a 43-kDa protein in human liver cytosol that binds specifically to regions within the 3′-UTR of CYP2A6 mRNA.

2. Materials and methods

2.1. Preparation of cytosol and microsomes

Normal human liver tissues obtained from the NCI Cooperative Human Tissue Network were used for the preparation of cytosols. Liver tissue was homogenized in 3 vol. of 0.25 M sucrose (pH 7.4), containing 1 mM EDTA and 25 mM HEPES at 4°. Cytosol was prepared as the final supernatant of centrifugation at 10,000 *g* (30 min, 4°) and 100,000 *g* (60 min, 4°) [32]. Microsomal pellets were resuspended in buffer [100 mM potassium phosphate (pH 7.4), 1 mM EDTA, 20% glycerol] and stored at –70° until further use. Mouse cytosol was prepared from livers of DBA/2N mice in a similar fashion.

2.2. Preparation of the DNA templates for in vitro transcription

A plasmid vector (pUC-19) containing the full-length cDNA for human CYP2A6 was provided by Dr. C.R. Wolf (Imperial Cancer Research Fund, University of Dundee). DNA templates for use in *in vitro* transcription of mRNA probes were produced by amplifying 3′-UTR of CYP2A6 by the PCR. DNA templates covered the full length of the 3′-UTR (257 bp); in addition, progressively truncated sequences (50 bp, 117 bp, 182 bp) were generated in order to more precisely map the binding sequence (see Fig. 1). Typical PCR reaction mixtures included CYP2A6 template cDNA (1 μg), oligonucleotide primers (33 pM), 200 μM dNTP, 1.5 mM MgCl, 10X PCR buffer and 2.5 U Taq polymerase (Promega Corp.) in a total reaction volume of 100 μL. The PCR reaction conditions were as follows: 1 min at 94°, 30 sec at 54°, and 1 min at 72° for 30 cycles. The same sense primer was used to amplify template DNA for the synthesis of all truncated RNA probes. This primer included a T7 RNA polymerase promoter sequence, which is underlined:

5′-TAATACGACTCACTATAGGGTGAGCGAGGGCTGTGCCGGT-3′

The antisense primer sequences and primer lengths were as follows:

Full length probe: (nts 1478–1734; 257 bp);
Antisense primer: 5′-CAGGAAATAAGAGCT-3′
Truncation probe 1: (nts 1478–1659; 182 bp);
Antisense primer: 5′-GAAGCACCTTATCAAGGTGAA-3′
Truncation probe 2: (nts 1478–1594; 117 bp);
Antisense primer: 5′-CTTCCTTTCCGCCATCCTGCCCCCAGT-3′
Truncation probe 3: (nts 1478–1527; 50 bp);
Antisense primer: 5′-CCTTTCCCTGGCCCCGCCCAC-3′

The DNA template for non-specific RNA used in competition experiments represented a 167 mer fragment derived from the multiple cloning site of pBS SK (+/–) plasmid (Stratagene Inc.), which was also produced by PCR in a similar manner. PCR-amplified DNA fragments were run on 2% agarose gels and visualized by ethidium bromide staining. DNA templates were subsequently used for *in vitro* transcription.

2.3. In vitro transcription

Radiolabeled and unlabeled mRNA fragments were transcribed *in vitro* with T7 RNA polymerase from PCR-amplified CYP2A6 and pBS cDNA templates as described [33]. *In vitro* transcription was performed in the presence of 1 μg of template DNA, 60 μCi of [α-^{32}P]UTP (800 Ci/mmol; Amersham-Pharmacia Biotech), unlabeled rGTP, rCTP, rATP (2.5 mM), 10 mM dithiothreitol, RNasin (20 U), and T7 RNA polymerase (2 U) at 37° for 60 min. Template DNA was digested with 1 U of RNase-free DNase, and unincorporated nucleotides were removed by

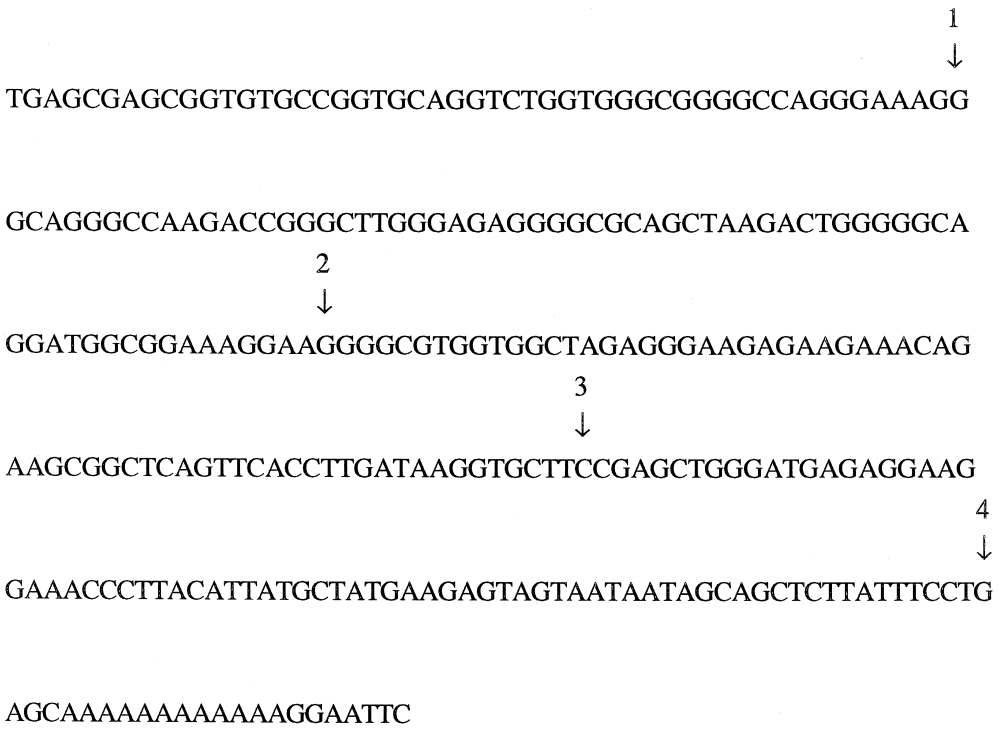

Fig. 1. Sequence of the entire 3′-UTR of CYP2A6 mRNA. Full-length and truncated probes corresponding to various parts of the CYP2A6 3′-UTR are indicated. From the 5′ end they finish at: 1 for the 50 nt, 2 for the 117 nt, 3 for the 182 nt, and 4 for the 257 nt probes. For further details, see "Materials and methods."

centrifugation using a centricon 30 membrane (Amicon Inc.). [^{32}P]UTP-labeled RNA was quantified by liquid scintillation counting, diluted to 60,000 cpm/μL, and used in EMSA.

2.4. EMSA

EMSAs were performed as previously described [34]. Briefly, cytosolic protein (50 μg) was incubated with radiolabeled CYP2A6 mRNA (60,000 cpm) in 10 μL of a solution containing 10 mM HEPES, 25 mM KCl, 10% glycerol, 1 mM dithiothreitol at 30^{o} for 15 min. After completion of the binding reaction, 20 U of RNase T1 (Pharmacia Biotech) and heparin sulfate (final concentration of 5 mg/mL) were added sequentially to the reaction mixture and incubated for 10 min, each at 30^{o}. For competition experiments, unlabeled CYP2A6 or plasmid RNA was added, in molar excess, to the cytosol 10 min before the addition of radiolabeled RNA. In some experiments, the binding reaction was treated with proteinase K (Boehringer-Mannheim) to a final concentration of 2 mg/mL for 30 min at 37^{o}. Following a 15-min incubation at 30^{o}, 2 μL of 6X native gel loading buffer (30% glycerol, 0.025% bromphenol blue, and xylene cyanol) was added, and the RNA–protein complexes were resolved on an 8% native polyacrylamide gel with 0.5X TBE buffer. Gels were pre-electrophoresed for 30 min at 20 mA followed by electrophoresis of RNA–protein complexes for 2 hr at 30 mA. Gels were dried and subjected to autoradiography for 10–20 hr.

2.5. UV cross-linking and electrophoresis of RNA–protein complexes

Binding reactions were performed as described above. After binding was completed, the samples were treated with 20 U of RNase T1 and then exposed to UV light in a Stratalinker UV light box, model 1800 (Stratgene) at 250 μJ/cm^2 for two cycles. The reaction products were denatured by boiling for 3 min in SDS loading buffer and resolved on an 8% SDS–polyacrylamide gel under non-reducing conditions. High resolution molecular mass markers (Broad Range Protein Markers, New England Biolabs) covering a range of 2–212 kDa were used to establish the molecular mass of the binding protein.

2.6. Western blot analysis

Microsomal protein (50 μg) was separated by 8% SDS–PAGE, transferred to nitrocellulose, and incubated with polyclonal antibodies raised against purified human hepatic CYP2A6 (Gentest Corp.). Following incubation with goat anti-rabbit peroxidase conjugated secondary antibody, bands on blots were detected by chemiluminescence (ECL, Amersham-Pharmacia Biotech) and visual-

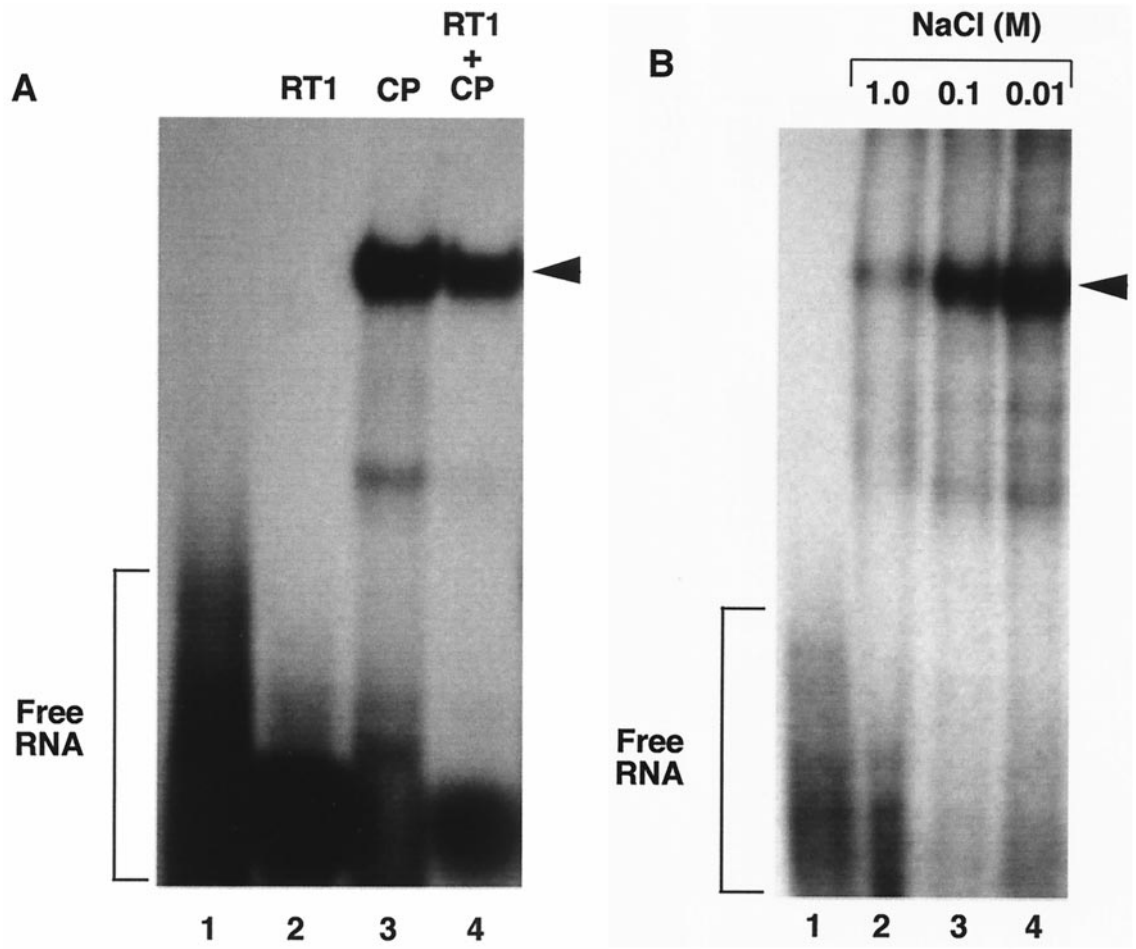

Fig. 2. Interaction of a *trans*-acting factor with the 3′-UTR of CYP2A6 mRNA. (A) Effect of RNase T1 treatment on complex integrity. Lane 1, CYP2A6 3′-UTR mRNA probe without RNase T1 (RT1) treatment; lane 2 probe with RNase T1 treatment. Note that addition of cytosolic protein (50 μg) from human liver to the labeled RNA resulted in a band shift (lane 3) that remained intact after the addition of RNase T1 (lane 4). (B) Effect of ionic strength on CYP2A6 mRNA–protein complex formation. Note that complex formation was progressively reduced in the presence of increasing salt concentrations: 1.0 M (lane 2), 0.1 M (lane 3), or 0.01 M (lane 4) NaCl. Protein concentration in the binding reaction was 5.0 mg/mL. The complex is indicated by an arrow in both panels.

ized by autoradiography. The relative concentration of CYP2A6 in different samples was then determined by densitometry.

3. Results

3.1. Identification of a cytosolic protein forming a complex with the 3′-UTR of CYP2A6 mRNA

To determine if a cytosolic protein(s) specifically interacts with the 3′-UTR of CYP2A6 mRNA, we incubated cytosol from human or mouse liver with an *in vitro* transcribed, ^{32}P-labeled probe covering nucleotides 1478–1734 of the 3′-UTR of CYP2A6 mRNA. Products of binding reactions were then analyzed by EMSA on native, low ionic strength (0.5X TBE) polyacrylamide gels (8%). Addition of cytosolic protein retarded the migration of the CYP2A6 mRNA probe (Fig. 2A). The complex was resistant to RNase T1 digestion (Fig. 2A, lane 4). When complex formation was carried out in the presence of NaCl of increasing ionic strength, dissociation of complexes occurred only at high salt concentrations (Fig. 2B).

Several observations suggested that formation of the RNA complex was due to protein binding. The band representing the RNA–protein complex was abolished following treatment of the binding reaction with proteinase K (Fig. 3A, lane 2). Furthermore, protein denaturation by pretreatment of cytosol at 56^{o} for 10 min (Fig. 3A, lane 3) inhibited binding activity completely. Complex formation was proportional to the amount of cytosolic protein from human liver (Fig. 3B) included in the binding reaction.

3.2. Importance of RNA structure in mRNA–protein interaction

Several protein–RNA interactions depend upon “stem-loop” formations by complementary bases within the

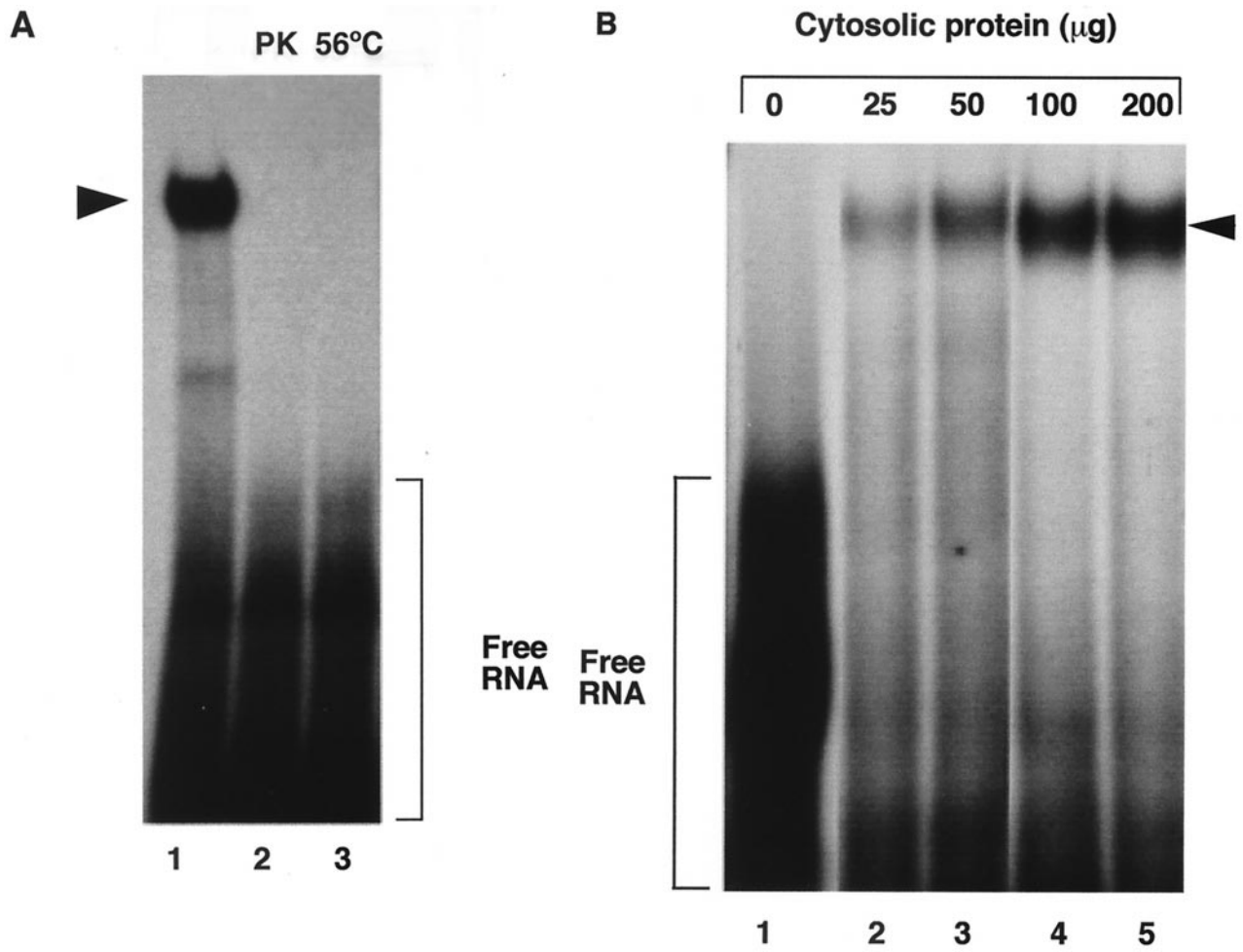

Fig. 3. Identification of the *trans*-acting factor as a cytosolic protein. (A) Effect of treatment with proteinase K (PK; lane 2) or prior heat denaturation (56°) of cytosolic protein from human liver (lane 3). (B) Effect of the amount of cytosolic protein from human liver on CYP2A6 mRNA complex formation. Amounts of cytosolic protein are shown. The complex is indicated by an arrow in both panels.

3′-UTR. To determine whether the secondary structure of the RNA is important in the interaction with the protein, the RNA probe was first heat denatured at 90° for 5 min and then was either cooled down rapidly to 4° to maintain the denatured structure, or cooled down gradually to room temperature to allow the secondary stable structure of the RNA to reform. Gel shift experiments were then conducted at 4°. Formation of complexes was similar with all probes (Fig. 4), indicating that an RNA secondary structure is not required for protein interactions.

3.3. Determination of molecular mass of binding protein

To determine the size of CYP2A6 mRNA–protein complexes, products of binding reactions were UV cross-linked and analyzed on 8% SDS polyacrylamide gels. A ^{32}P-labeled protein band with a molecular mass of approximately 43 kDa was resolved with cytosol from both human (lane 1) and mouse (lane 2) liver (Fig. 5) and the full length 3′-UTR CYP2A6 probe.

3.4. Localization of the binding site within the 3′-UTR of CYP2A6

To more precisely define the minimal cognate sequence within the 3′-UTR of CYP2A6 to which the 43-kDa cytosolic protein binds, RNA binding studies were conducted with RNA probes consisting of the full-length 3′-UTR sequence and truncated probes consisting of sequentially shorter segments of CYP2A6 3′-UTR (Fig. 6). RNA–protein complexes occurred with all truncated RNA probes, indicating that the binding site was located within a 50 nucleotide sequence between nucleotides 1478 and 1527 of the CYP2A6 3′-UTR.

3.5. Specificity of RNA–protein interaction

The specificity of the CYP2A6–RNA interaction was assessed in competition experiments with unlabeled specific and non-specific RNA. Cytosols were preincubated with increasing amounts of unlabeled CYP2A6 mRNA probe or non-specific pBS RNA before being incubating with labeled

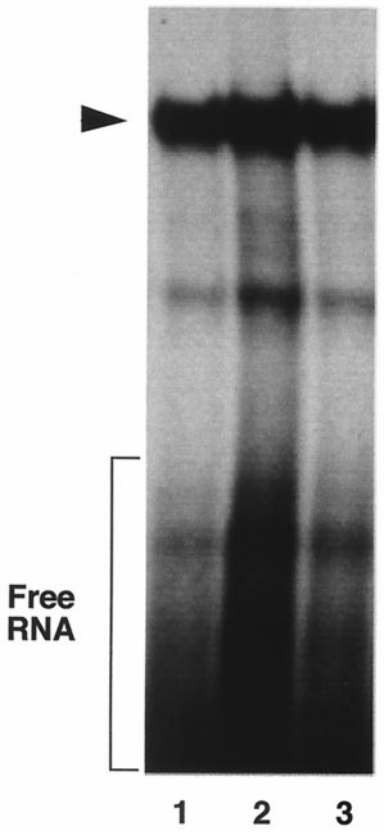

Fig. 4. Influence of RNA secondary structure on complex formation. Binding reaction was performed at 4° with unheated CYP2A6 probe (lane 1), or after the probe had been preheated to 90° for 10 min and then either cooled rapidly to 4° (lane 2) or cooled gradually to room temperature (lane 3). The concentration of cytosolic protein in the binding reaction was 0.5 mg/mL. The complex is indicated by an arrow.

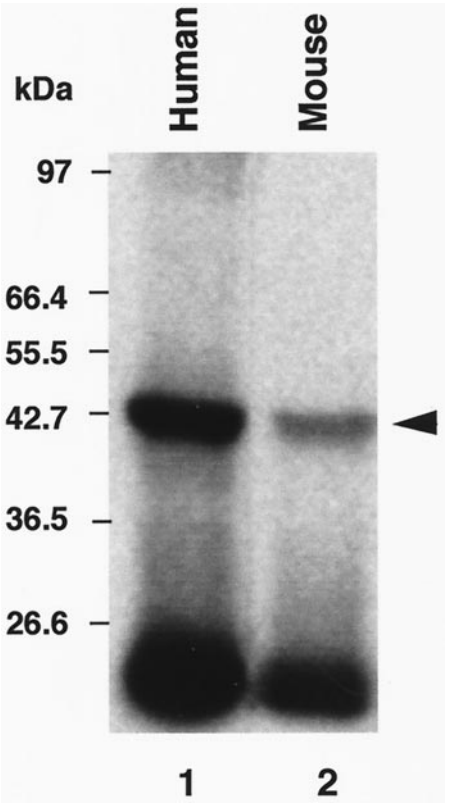

Fig. 5. Identification of CYP2A6 mRNA protein molecular mass by UV cross-linking. UV cross-linked complexes between CYP2A6 mRNA and cytosolic protein from human liver (lane 1) and mouse liver (lane 2) were visualized as major bands of approximately 43 kDa. Molecular mass markers are shown on the left. The complex is indicated by an arrow.

CYP2A6 mRNA. UV cross-linking experiments (Fig. 7A) show that competition occurred with unlabeled specific probe since CYP2A6 RNA–protein complex formation was reduced after the addition of 5 molar excess of unlabeled specific mRNA to the binding reaction (lane 4), and abolished after the addition of 10 molar excess (lane 5). In contrast, competition with the same concentration of non-specific plasmid RNA (pBS) had no effect on the binding activity (lanes 2 and 3). Competition experiments with the smallest truncated RNA probe (50 nt) produced similar results, indicating that binding specificity was retained with the minimal binding sequence (Fig. 7B).

3.6. Comparision of RNA–protein complex formation with levels of CYP2A6 mRNA and microsomal protein

To begin to examine the biological significance of CYP2A6 mRNA–protein complex formation with respect to hepatic CYP2A6 expression, levels of CYP2A6 protein were compared to complex formation between the full-length CYP2A6 mRNA probe and hepatic cytosolic protein from the same individuals (Fig. 8). Considerable variation in CYP2A6 microsomal protein expression and the degree of RNA–protein complex formation was observed between individuals. Comparison of the degree of RNA–protein complex formation and CYP2A6 protein by densitometry (Fig. 8C) and by regression analysis ($r^2 = 0.8$, data not shown) suggested that RNA–protein interactions may play a role in CYP2A6 regulation.

4. Discussion

The results of this study demonstrate a specific interaction between a 43-kDa cytosolic protein from human liver and a *cis* element located between nucleotides 1478 and 1527 of the 3′-UTR of CYP2A6 mRNA. Formation of this complex is highly specific and occurs even in the presence of a 10-fold molar excess of non-specific competitor mRNA. The RNA–protein interaction is strong, as complex formation was reduced only in the presence of high salt concentration. Elimination of the complex with proteinase K illustrates that the binding factor is at least partially composed of protein, but does not exclude the possibility that there are also non-protein components.

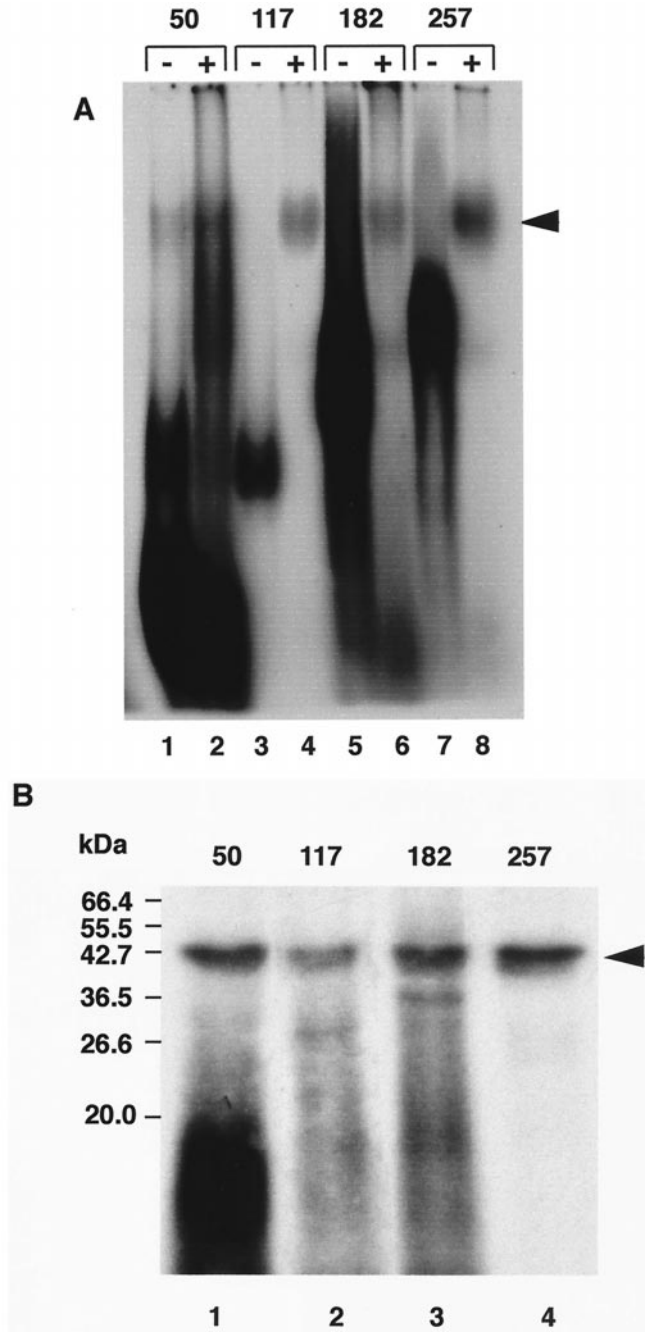

Fig. 6. Mapping of the minimal binding region within the CYP2A6 3′-UTR required for protein binding. RNA–protein complexes formed with human liver cytosolic protein and all truncated RNA probes as illustrated by EMSA (A) and UV cross-linking analysis (B). Probe lengths are indicated at the top of each lane. The presence or absence of cytosolic protein in the binding reaction mixture is denoted as (+) and (–), respectively, in panel A. Molecular mass markers are shown on the left of panel B. The complex is indicated by an arrow in both panels.

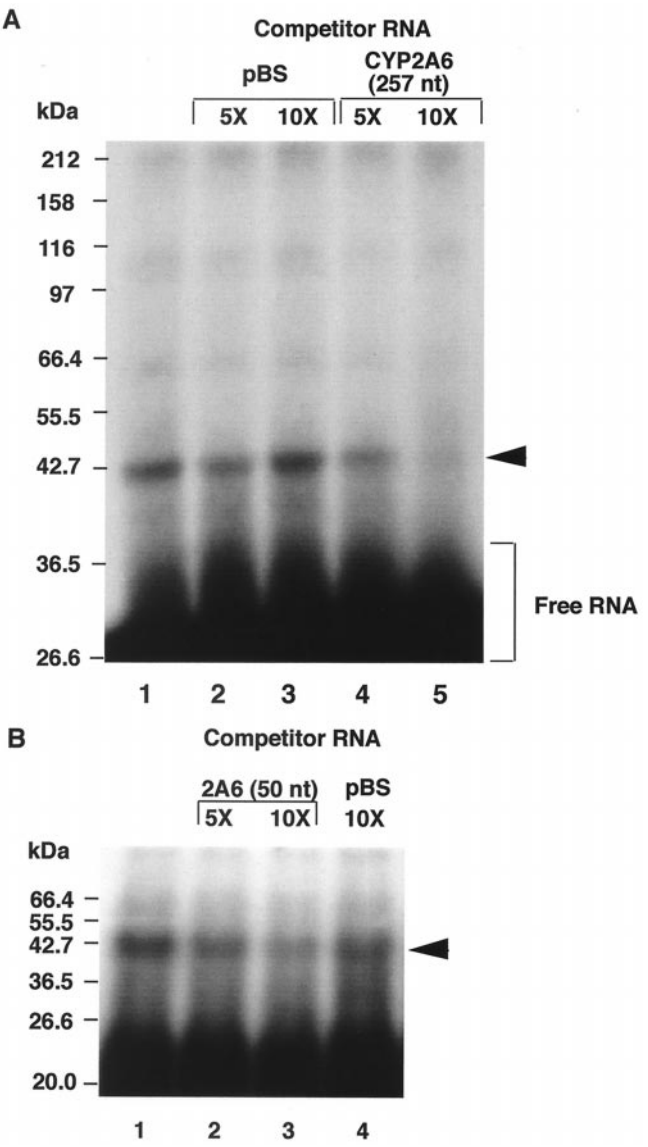

Fig. 7. UV cross-linking analysis of RNA–protein interactions in the presence of competitor RNAs. (A) Competition assays using the 257-nt probe (full length 3′-UTR). Note that the 43-kDa band (lane 1) was reduced by the addition of a molar excess of unlabeled CYP2A6 mRNA (lanes 4 and 5) but was unaffected by unrelated plasmid (pBS SK+/–) mRNA (lanes 2 and 3). (B) Competition assays using the 50-nt probe. Note that binding specificity was maintained with shorter fragments containing the binding sequence. Molecular mass markers are shown on the left. The complex is indicated by an arrow in both panels.

UV cross-linking experiments indicate that complexes also occur between CYP2A6 3′-UTR mRNA and a 43-kDa protein in mouse hepatic cytosol. Recently, a pyrazole-inducible 44-kDa protein was identified by UV cross-linking, which binds to a 70-nt adenosine/uridine-rich region of the 3′-UTR of Cyp2a5 mRNA [29]. Several other pyrazole-inducible proteins with molecular masses of 45/48, 37/39, and 70/72 kDa, which bind specifically to this 70-nt region, have also been identified [30]. Of these, a 37/39 kDa RNA-binding protein has been partially characterized, and there is some indication that it may be closely related to the hnRNP A1, an RNA-binding protein involved in gene regulation [31]. The relationship of the 43-kDa CYP2A6-mRNA-binding protein described in this study to the several proteins that bind to Cyp2a5 mRNA is unclear. Further protein characterization, including purification and N-terminal

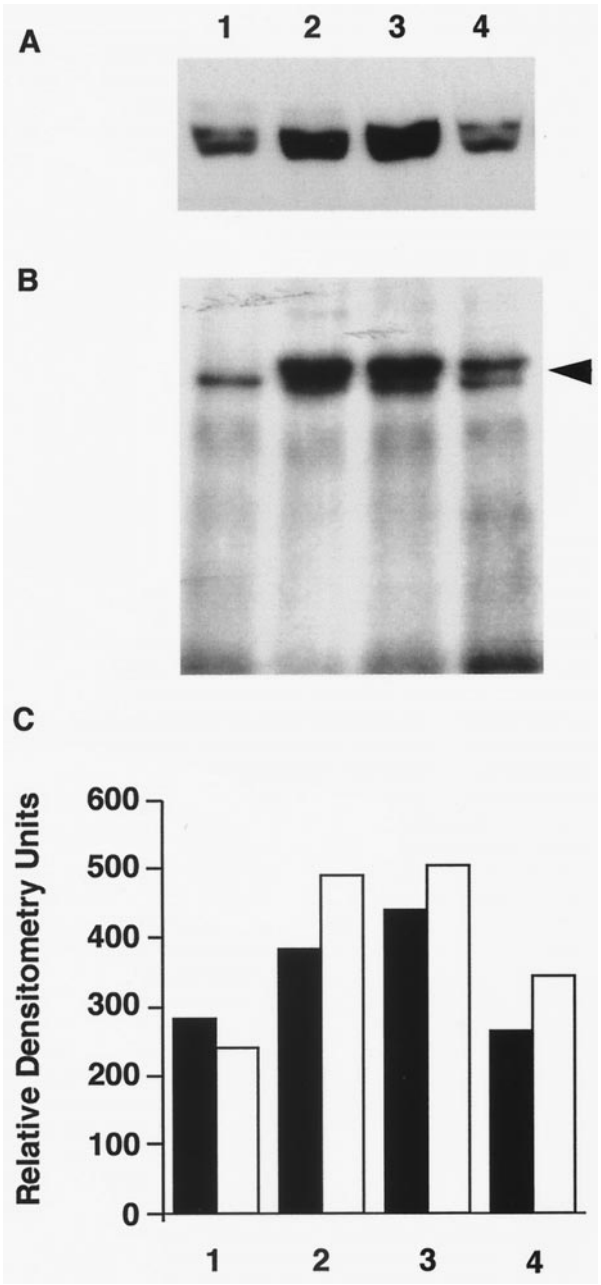

Fig. 8. Comparison of RNA–protein complex formation with CYP2A6 microsomal protein levels in human liver. (A) Immunoblot analysis of CYP2A6 in human liver microsomes from various individuals showing inter-individual variation in expression. (B) EMSA of complex formation with hepatic cytosolic protein from individuals depicted in panel A and full-length CYP2A6 3′-UTR probe. Note the variation in complex formation and the correlation of the intensity of complex bands with the amount of CYP2A6 microsomal protein. Equivalent amounts of cytosolic protein (50 μg) were included in each binding reaction mixture. The complex is indicated by an arrow. (C) Densitometric quantitation of RNA–protein complex and CYP2A6 microsomal protein formation shown in panels A and B. Black bars: CYP2A6 protein expression. White bars: RNA–protein complex formation.

amino acid sequencing, is necessary to clarify any possible relationship.

We have mapped the primary binding site of the 43-kDa protein to a 50-nt region at the beginning of the CYP2A6 3′-UTR. This binding site has not been identified previously since our computer analysis of the CYP2A6 3′-UTR failed to reveal complementarity with sequences for binding sites of known RNA-binding proteins. Furthermore, there is no evidence of secondary structures within the 3′-UTR of CYP2A6 RNA that might influence protein binding. Indeed, the observation that heat denaturation of the CYP2A6 RNA probe did not inhibit complex formation suggests that the 43-kDa RNA-binding protein does not recognize two-dimensional structural domains. Recently, the primary binding site of the 37/39 kDa, pyrazole-inducible Cyp2a5 mRNA binding protein was mapped to a UAGGA motif at the tip of a 71-nt hair-pin loop in the 3′-UTR [31]; however, this sequence is not present in the 3′-UTR of CYP2A6 mRNA.

Correlation between the level of CYP2A6 microsomal protein and 3′-UTR–protein complex formation suggests a potential role of the RNA binding protein as a determinant of CYP2A6 expression; however, the influence of protein–RNA interactions in modulating CYP2A6 mRNA stability was not specifically addressed. Previous studies have demonstrated marked variation (up to 80-fold) in CYP2A6 expression and activity in various human liver samples [3,35]. In mice, induction of Cyp2a5 by pyrazole has been well-characterized and occurs through a post-transcriptional mechanism involving message stabilization [21,22]. Because pyrazole treatment increases both Cyp2a5 mRNA levels and Cyp2a5 3′-UTR–protein complex formation, it has been suggested that this protein may play a role in RNA stabilization; however, this has not been confirmed [29]. Increases in RNA–protein complex formation could be due to either induction of binding protein expression or increased affinity of the binding protein to the *cis*-acting element. While our results suggest that protein–RNA interactions may be the basis for the post-transcriptional control of CYP2A6 expression, further studies examining the influence of CYP2A6 3′-UTR RNA–protein complexes on RNA stability in reporter gene constructs are required to confirm this possibility.

Current efforts to understand the regulatory control of CYPs have focused mainly on constitutive and inducible transcription of CYP genes and associated DNA elements [36–38], whereas post-transcriptional mechanisms of CYP regulation have received less attention. Factors that modulate degradation of pre-existing mRNA and differences in the rates of mRNA decay may explain inter-individual variability in the expression of various drug-metabolizing enzymes. For example, variability in glutathione *S*-transferase Pi mRNA levels due to altered cellular redox [39], diurnal variations of CYP7A1 mRNA levels [40], changes in CYP2E1 mRNA levels during fasting and uncontrolled diabetes [41,42], and temporal patterns of CYP1A1/CYP1A2 mRNA expression after induction with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [43] have been attributed to differences in mRNA decay rates.

In addition, two proteins that bind to a poly(U) region of CYP1A2 have been identified; however, details about their relationship to the regulation of CYP1A2 expression are not available [44].

In conclusion, we have demonstrated, by EMSA, the presence in human liver of a 43-kDa cytosolic protein that binds specifically to the 3′-UTR of CYP2A6 mRNA. While more work is needed to characterize the protein and the functional role of RNA–protein interactions, these findings have potentially important implications with respect to regulation of CYP2A6 gene expression and lay the foundation for future detailed studies on the regulatory mechanisms of CYP2A6.

Acknowledgments

We thank Dr. C.R. Wolf (Imperial Cancer Research Fund, University of Dundee) for providing human CYP2A6 cDNA. This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada.

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