Identification and characterization of a 44 kDa protein that binds specifically to the 3'-untranslated region of CYP2a5 mRNA: inducibility, subcellular distribution and possible role in mRNA stabilization

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Stabilization of mRNA is important in the regulation of CYP2a5 expression but the factors involved in the process are not known [Aida and Negishi (1991) *Biochemistry* **30**, 8041–8045]. In this paper, we describe, for the first time, a protein that binds specifically to the 3'-untranslated region of CYP2a5 mRNA and which is inducible by pyrazole, a compound known to increase the half-life of CYP2a5 mRNA. We also demonstrate that pyrazole treatment causes an elongation of the CYP2a5 mRNA poly(A) tail, and that phenobarbital, which is transcriptional activator of the CYP2a5 gene that does not affect the mRNA half-life, neither induces the RNA-binding protein nor affects the poly(A) tail size. SDS/PAGE of the UV-cross-linked RNA–protein complex demonstrated that the RNA-binding protein

has an apparent molecular mass of 44 kDa. The protein-binding site was localized to a 70-nucleotide region between bases 1585 and 1655. Treatment of cytoplasmic extracts with an SH-oxidizing agent, diamide, an SH-blocking agent, *N*-ethylmaleimide or potato acid phosphatase abolished complex-formation, suggesting that the CYP2a5 mRNA-binding protein is subject to post-translational regulation. Subcellular fractionation showed that the 44 kDa protein is present in polyribosomes and nuclei, and that its apparent induction is much stronger in polyribosomes than in nuclear extracts. We propose that this 44 kDa RNA-binding protein is involved in the stabilization of CYP2a5 mRNA by controlling the length of the poly(A) tail.

INTRODUCTION

The cytochrome *P*-450 mono-oxygenases (CYPs) comprise a large and diverse family of enzymes [1] responsible for the initial oxidation of various lipophilic compounds, including xenobiotics and endogenous substrates. The xenobiotic-metabolizing isoenzymes are often inducible, and several mechanisms are involved in the regulation of their expression. These include gene transcription, pre-mRNA processing, mRNA stabilization, translational efficiency and protein stabilization [2]. Perhaps the best characterized is the transcriptional regulation of CYP1A1 [3,4]; a ligand-bound aryl hydrocarbon receptor complexed with an aryl hydrocarbon receptor nuclear translocator [5] binds to the xenobiotic-responsive element in the upstream region of the *CYP1A1* gene and induces transcription of *CYP1A1* [6,7].

Although several CYP isoenzymes are regulated post-transcriptionally, only very little is known about the molecular mechanisms involved in the process. CYP2a5, which catalyses xenobiotic oxidation [8,9], is one of the isoenzymes for which post-transcriptional control is essential. This enzyme has been purified and cloned from mouse liver [10–12] and its expression is enhanced by several structurally diverse xenobiotics such as pyrazole, phenobarbital, hexachloro-1,3-butadiene, cobalt and cocaine [10,13–16]. Pyrazole, which is perhaps the strongest and most selective inducer of CYP2a5, has been found to increase its expression by mRNA stabilization; the mRNA half-life is approx. 1.5 h in control and approx. 6 h in pyrazole-treated mice [13,17]. The increase in CYP2a5 mRNA half-life is known to be associated with a large increase in hepatic CYP2a5 mRNA and protein levels, as well as with increased enzyme activity but the molecular mechanism governing the post-transcriptional regulation is not understood [10,16].

Several studies have shown that the poly(A) tail plays a major role in the control of mRNA stability [18]. Specific sequences

have been identified within the 3'-untranslated region (3'-UTR) that interact with *trans*-acting factors (regulatory proteins) which may affect the half-life of the mRNA [19,20]. In this respect the iron-responsive element in the 3'-UTR of transferrin receptor mRNA and the corresponding binding protein constitute the best-characterized example of the control of mRNA half-life [21]. Binding of the iron-responsive element binding protein to the iron-responsive element is up-regulated by low iron concentration and has a stabilizing effect on the mRNA [22–25]. Another example of the control of mRNA half-life by specific elements is the AUUUA instability motifs found in the 3'-UTR of many short-lived mRNAs including those encoding proto-oncogenes, cytokines and lymphokines [26,27]. The existence of several *trans*-acting factors interacting with these AUUUA motifs has been reported.

To our knowledge, nothing has been reported on possible *trans*-acting factors that interact with the 3'-UTR of CYP mRNAs. In the present study, we have identified and characterized a protein from mouse liver that specifically binds to the 3'-UTR of CYP2a5 mRNA. We also show that increased binding of this protein to the mRNA is associated with increased mRNA stability and elongation of the CYP2a5 mRNA poly(A) tail. The possible role of the CYP2a5 3'-UTR-binding protein in the control of mRNA stability and processing is discussed.

EXPERIMENTAL

Animals

Male DBA/2N mice (6 weeks old) were obtained from IFFA Credo. They were either left untreated or injected intraperitoneally with pyrazole or phenobarbital dissolved in saline at doses of 200 or 80 mg/kg respectively for 3 consecutive days. The

Figure 1 CYP2a5 mRNA probes used for RNA–protein interactions

The diagram of the CYP2a5 cDNA shows the 5'-UTR, coding region and 3'-UTR. The oligonucleotides used to amplify different parts of CYP2a5 cDNA by PCR are indicated in parentheses (see Table 1). The number of nucleotides in the CYP2a5 portion of each RNA probe is given. The RNA probe ΔF corresponds to probe F but with a deleted AUUUA motif.

animals were killed 24 h after the last injection and the livers were removed.

Preparation of liver cytoplasmic extract

Excised mouse liver was homogenized with a Teflon/glass homogenizer in 15 mM Hepes buffer, pH 7.6, containing 3 mM $MgCl_2$, 40 mM KCl, 0.4% Nonidet P40, 0.5 mM PMSF and 10 $\mu g/ml$ leupeptin. The homogenate was centrifuged at 12000 *g* for 10 min at 4 °C. The supernatant was divided into aliquots and stored at –70 °C until used in RNA-binding assays. Protein concentration was determined by the Bradford method (Bio-Rad).

Subcellular fractionation

The S130 (cytosol) and polyribosomal fractions were prepared by the method of Brewer and Ross [28]. Briefly, liver was homogenized in buffer A [10 mM Tris/HCl, pH 7.6, 1.0 mM potassium acetate, 1.5 mM magnesium acetate, 2.0 mM dithiothreitol (DTT), 0.5 mM PMSF]. The homogenate was centrifuged at 12000 *g* for 10 min at 4 °C to remove the nuclei. The supernatant was layered over a cushion of buffer B [30% (w/v) sucrose, 10 mM Tris/HCl, pH 7.6, 1.0 mM potassium acetate, 1.5 mM magnesium acetate, 2.0 mM DTT, 0.5 mM PMSF], and centrifuged at 130000 *g* for 2.5 h, at 4 °C. The high-speed supernatant (S130) was removed and stored at –70 °C. The polyribosomal pellet was rinsed twice with buffer A, resuspended in the same buffer and stored at –70 °C. Liver nuclei were

purified by centrifugation through buffered 2 M sucrose [29], and nuclear extracts were prepared as described by Dignam et al. [30].

Northern-blot analysis

Total RNA was extracted using RNeasy Lysis solution [31] (Qiagen). For extraction of polyribosomal RNA, polyribosomes were prepared as described above except that buffers were made 10 mM with ribonucleoside–vanadyl complexes (Sigma) and RNA was extracted as described by Brewer and Ross [28].

RNA (20 μg) was electrophoresed through 1.2% formaldehyde/agarose gel and blotted on to Gene-Screen plus nylon filter (DuPont/NEN) and immobilized by UV fixation. The filter was hybridized with full-length CYP2a5 cDNA [13]. Full-length CYP2a5 cDNA containing plasmid was kindly provided by Dr. M. Negishi (LRDT, NIEHS, Research Triangle Park, NC, U.S.A.).

Preparation of radiolabelled RNAs

Using different sets of primers, segments of various parts of CYP2a5 cDNA were produced (see Figure 1, and Table 1). For assignment of nucleotide positions of primers, the CYP2a5 cDNA map as published by Squires and Negishi [12] was used. Sense oligonucleotides contained at the 5' end 23 nucleotides corresponding to the T7 RNA polymerase promoter. PCR-amplified products were transcribed with T7 RNA polymerase in

Table 1 Oligonucleotides used for PCR

Different sets of oligonucleotides were used to produce different parts of CYP2a5 cDNA. The T7 prefix indicates the T7 RNA polymerase promoter sequence which is added in order to permit the transcription of the PCR products. The sequence of the T7 RNA polymerase promoter is TAATACGACTCACTATAGGAGA. The site of the AUUUA motif deleted in P9 is indicated by Δ .

Oligonucleotide	Orientation	Sequence	Nucleotide/assignment
P1	Sense	T7-ACCTACACTATGAGTTTCTT	1456–1475
P2	Antisense	TGCCATAAATAATATCTACT	1670–1689
P3	Sense	T7-GTTTCGAGATTTCCTCCTCC	1136–1155
P4	Antisense	TGTAGGTTGGTGGGATCGTG	1443–1462
P5	Antisense	AATATCCTCTGCAAGTCTAC	1585–1604
P6	Sense	T7-GTAGACTTGCAGAGGATATT	1585–1604
P7	Antisense	ATTCCTATTGACAACATAGT	1635–1655
P8	Sense	T7-ACTATGTTGTCAATAGGAAT	1635–1655
P9	Antisense	ATTCCTATTGACAACATAGT Δ AT TCCCTCTTCTT	1618–1655

the presence of [α - 32 P]UTP (~ 800 Ci/mmol; Amersham) using standard protocols.

Transcription of *Eco*RI-digested pAU plasmid with T7 RNA polymerase produced an 80-base fragment containing four adjacent copies of the AUUUA motif [32]. pAU plasmid described by Malter and Hong [32] was kindly provided by Dr. James Malter, University of Wisconsin, Madison, WI, U.S.A.

Gel mobility shift assay

Binding reactions were performed essentially as described previously [33]; 10^5 c.p.m. of 32 P-labelled RNA and protein extracts (2–20 μ g) were incubated for 10 min at room temperature in 10 mM Hepes, pH 7.6, containing 3 mM $MgCl_2$, 40 mM KCl, 5% glycerol, 1 mM DTT and yeast tRNA (100 ng/ μ l). RNase T1 (20 units) was added and the reaction mixture was incubated for 20 min at room temperature. Electrophoresis of RNA-protein complexes was carried out on 7% native polyacrylamide gel (acrylamide/methylene bisacrylamide ratio, 37.5:1) with 50 mM Tris/glycine (pH 8.8) as running buffer. For competition experiments, unlabelled RNA was added before the addition of radiolabelled RNA. To determine the effects of diamide or *N*-ethylmaleimide (NEM), cytoplasmic extracts, buffer and tRNA were preincubated with diamide or NEM for 15 min at room temperature before the addition of 2-mercaptoethanol for 15 min (where indicated), followed by the addition of the RNA probe. To study the effect of dephosphorylation, cytoplasmic extract was preincubated with 3 μ g of potato acid phosphatase for 20 min at room temperature before the addition of the RNA probe. When indicated 2.5 μ g of proteinase K was added to the reaction mixture after RNase T1 digestion and the mixture was incubated at room temperature for 15 min.

UV-cross-linking assays

Binding reactions were carried out as for the gel mobility-shift assays. The reaction mixtures were then placed on ice and exposed to UV light for 5 min at a distance of about 10 cm in a stratalinker apparatus (Stratagene) (UV lamp BLE-8T312, Spectronics, Westbury, NY, U.S.A.). Mixtures were treated with 2 μ g of RNase A at 37 °C for 30 min to digest free RNA. Proteins were separated by a SDS/PAGE (12% gel) under non-reducing conditions.

Analysis of length of CYP2a5 mRNA poly(A) tail

The length of the CYP2a5 poly(A) tail was measured as described by Brewer and Ross [34], with minor modifications. Briefly, 20, 10 or 2 μ g of RNA from control, phenobarbital or pyrazole-treated mice respectively was incubated with 400 ng of oligonucleotide P4 (see Table 1) either alone or together with 400 ng of oligodT₂₀ (Pharmacia). RNase H digestion (1 unit) was performed at 37 °C for 60 min, followed immediately by the addition of 60 μ l of sterile water and 1 μ l of glycogen (Boehringer). The samples were extracted with phenol/chloroform, electrophoresed through 2.5% agarose/formaldehyde gels, blotted on to the gene screen plus nylon filter and fixed with UV. The filter was then hybridized with a cRNA probe specific for the CYP2a5 3'-UTR.

RESULTS

Inducible binding of a cytoplasmic protein to the 3'-UTR of CYP2a5 mRNA

To determine whether *trans*-acting factors that interact with CYP2a5 mRNA exist, we performed gel mobility-shift assays with radiolabelled CYP2a5 mRNA fragments of various lengths (Figure 1). Since mRNA-protein interaction often takes place within the 3'-UTR of mRNA [19,20], we first examined the ability of liver cytoplasmic proteins to bind to an mRNA probe corresponding to the entire 3'-UTR of CYP2a5 (Figure 1, probe A). Figure 2 shows that the addition of cytoplasmic extract to a

Figure 2 Binding of a pyrazole-inducible protein to the 3'-UTR of CYP2a5 mRNA

RNA gel mobility-shift assays were carried out as detailed in the Experimental section. Complex-formation was performed with RNA probe A (Figure 1) in the absence of cytoplasmic extract (lane 1) or in the presence of cytoplasmic extract (20 μ g of protein) from control (lane 2) or pyrazole-treated liver (lane 3). Treatment of the mixture before electrophoresis with proteinase K abolished the band (lane 4).

Figure 3 Determination of the apparent molecular mass of the binding protein by UV-cross-linking

RNA probe A was incubated in the absence of cytoplasmic extract (lane 1), or in the presence of cytoplasmic extract (20 μ g of protein) from control (lane 2) or pyrazole-treated (lanes 3–5) liver. The preparations were either cross-linked by UV irradiation as described in the Experimental Section (lanes 1, 2, 4 and 5) or incubated on ice (lane 3). Treatment with proteinase K (lane 5) prevented the appearance of the RNA–protein complex. Molecular-mass standards are indicated (kDa).

mixture containing probe A resulted in a gel-shifted band (lane 2) and that the intensity of this band was substantially increased when liver cytoplasmic extract from pyrazole-treated mice was used (lane 3 compared with lane 2). Treatment of the binding reaction mixture with proteinase K completely abolished the shifted band (lane 4). These results indicate that the binding factor is a protein that is inducible by pyrazole, a xenobiotic that increases the half-life of CYP2a5 mRNA.

Determination of the molecular mass and binding specificity of the CYP2a5 mRNA-binding protein

To determine the apparent molecular mass of the CYP2a5 mRNA 3'-UTR-binding protein, UV-cross-linking assays were performed. As shown in Figure 3, one major RNA–protein complex of approx. 44 kDa was resolved (lane 4). An additional complex of approx. 75 kDa was occasionally seen. Lanes 1 and 3 show that in the absence of extract or in the absence of UV irradiation no complexes were seen. The UV-cross-linking experiments also demonstrate the inducibility of the 44 kDa band by pyrazole (Figure 3, lanes 2 and 4) and its proteinaceous nature (lane 5) and therefore suggest that the 44 kDa band represents the protein detected in Figure 2.

To examine the sequence specificity of the mRNA–protein complex-formation, we carried out competition gel mobility-shift assays. A 32 P-labelled 3'-UTR RNA was used as a probe (Figure 1, probe A) and several unlabelled mRNAs were tested for their ability to compete for the binding. Figure 4(A) shows that a large excess of yeast tRNA does not prevent complex-formation (lanes 1–3), neither does the unlabelled coding region of CYP2a5 mRNA (Figure 1, probe C) (Figure 4A, lanes 4–6). In contrast, the 3'-UTR mRNA (probe A) competed efficiently for binding (lanes 7–9). These results indicate a binding specificity for the 3'-UTR of CYP2a5 mRNA. To examine further the binding specificity, competition assays were performed with each homoribopolymer (Figure 4B). None of these homoribopolymers were able to prevent efficiently complex-formation, demonstrating that the binding protein does not recognize homopolymeric strands and suggesting that binding of the protein to its recognition sequence in the CYP2a5 3'-UTR is highly sequence specific. Competition experiments using UV-cross-linking assays

Figure 4 Specificity of the RNA–protein complex-formation

(A) RNA gel mobility-shift assays were carried out in the presence of 0.5, 1 or 2 μ g of unlabelled yeast tRNA (lanes 1–3 respectively), 50, 100 or 250 ng of unlabelled CYP2a5-coding region RNA (probe C, Figure 1) (lanes 4–6 respectively) or 50, 100 or 250 ng of unlabelled CYP2a5 3'-UTR RNA (probe A, Figure 1) (lanes 7–9 respectively). (B) Gel mobility-shift assays were performed in the presence of 100 ng (lanes 1, 4, 7 and 10), 250 ng (lanes 2, 5, 8 and 11) and 500 ng (lanes 3, 6, 9 and 12) of poly(rA) (lanes 1–3), poly(rU) (lanes 4–6), poly(rG) (lanes 7–9) and poly(rC) (lanes 10–12). (C) UV-cross-linking assays were carried out without competitor (lane 1) or with 100 ng (lanes 2, 5, 8 and 11), 250 ng (lanes 3, 6, 9 and 12) or 500 ng (lanes 4, 7, 10 and 13) of poly(rA) (lanes 2–4), poly(rU) (lanes 5–7), poly(rG) (lanes 8–10) and poly(rC) (lanes 11–13). Unlabelled coding region RNA (probe C) (lane 14) and 3'-UTR (probe A) (lane 15) (200 μ g of each) were also used as competitors.

indicated the same binding specificity for the 3'-UTR of CYP2a5 mRNA as was obtained with the non-denaturing gels (Figure 4C).

Localization of the protein-binding site within the 3'-UTR

Several RNA probes were prepared to localize the protein-binding site within the transcript (Figure 1, probes A–G). The probes were tested by UV-cross-linking assays and the results

Figure 5 Protein-binding site in CYP2a5 mRNA

(A) Various RNA probes described in Figure 1 (probes A–G) were tested in UV-cross-linking assays (lanes 1–7 respectively). Probe F located between positions 1585 and 1655 in the 3'-UTR of CYP2a5 mRNA contains the binding site (lane 6). (B) The sequence of RNA probe F is given. (C) UV-cross-linking assays were performed using an RNA probe containing four adjacent AUUUA motifs (see the Experimental section) (lanes 1 and 2), RNA probe F (lanes 3, 4, 5, 7, 8 and 9) and an RNA probe ΔF corresponding to probe F but with a deleted AUUUA motif (see the Experimental section) (lane 6). Incubations were carried out with cytoplasmic extract (20 μ g of protein) from control (lanes 1 and 3) or pyrazole-treated (lanes 2, 4, 5, 6, 7, 8, 9) liver. Four unlabelled AUUUA containing RNAs (250 ng) (lane 5) and 50, 100 and 200 ng of unlabelled RNA probe ΔF (lanes 7–9 respectively) were used as competitors.

obtained (Figure 5A) further confirmed that the binding site is within the 3'-UTR (lanes 1–3). An RNA fragment corresponding to 70 nucleotides, located between positions 1585 and 1655 in the 3'-UTR of CYP2a5 mRNA, was identified as containing the protein-binding site (Figures 1, fragment F; for the sequence see Figure 5B). Since this site contains one AUUUA motif previously found in different mRNAs and is known to interact with several proteins [35,36], we investigated the potential role of the motif in complex-formation (Figures 5C). UV-cross-linking assays using an RNA probe containing four adjacent AUUUA motifs (see the Experimental section) showed that mouse liver cytoplasmic extract contains a protein that interacts with these motifs (Figures 5C, lanes 1 and 2). However, this protein has an apparent molecular mass of 40 kDa compared with the molecular mass (44 kDa) of the protein that interacts with CYP2a5 3'-UTR (Figures 5C, lanes 1 and 2 compared with lane 4), suggesting that these two proteins are different. In addition, it was found that pyrazole treatment does not induce the AUUUA-binding protein (lanes 1 and 2 compared with lanes 3 and 4) and that an excess of four unlabelled AUUUA-containing RNAs did not prevent CYP2a5 3'-UTR-protein complex-formation (lane 5). Furthermore, a UV-cross-linking assay with probe ΔF , which corresponds to RNA probe F but with the AUUUA motif deleted (see Figure 1), revealed the same 44 kDa protein as probe F (lane 6).

Figure 6 Biochemical characterization of the CYP2a5 mRNA 3'-UTR binding protein

(A) Gel mobility-shift assays were performed with cytoplasmic extract (20 μ g of protein) from pyrazole-treated liver preincubated with the buffer only (lane 1), with 10 mM NEM (lane 2) or 10 mM diamide (lanes 3–6). Cytoplasmic extracts preincubated with 10 mM diamide were then treated with 0.5, 1 or 2% 2-mercaptoethanol (lanes 4–6 respectively) before addition of the RNA probe. (B) RNA gel mobility-shift assays were carried out with cytoplasmic extract from pyrazole-treated liver preincubated for 20 min with buffer only (lane 1) or potato acid phosphatase (lane 2).

Finally, complex-formation with probe F was prevented by an excess of unlabelled probe ΔF (Figure 5C, lanes 7–9).

Taken together these data demonstrate that the recognition sequence in the CYP2a5 3'-UTR for the 44 kDa inducible protein is not the AUUUA motif and that CYP2a5 3'-UTR-protein complex-formation is independent of AUUUA motifs.

Biochemical characterization of the CYP2a5 mRNA 3'-UTR-binding protein

It has been reported that proteins involved in the control of mRNA half-life are often post-translationally modified to contain thiol and phosphate groups which are important for their function and critical for their binding to mRNA [32,37]. We wanted to know whether this is also the case for the protein binding to CYP2a5 3'-UTR. For this purpose, we examined the effects of a thiol-alkylating agent (NEM) and a thiol-oxidizing agent (diamide) on mRNA binding. Pretreatment of cytoplasmic extract with 10 mM NEM or 10 mM diamide abolished the RNA-binding activity (Figures 6A, lanes 2 and 3) which could partially be restored after diamide treatment by incubation with 0.5–2.0% 2-mercaptoethanol (lanes 4–6). In addition, the CYP2a5 3'-UTR-binding activity was abolished by pretreatment of the cytoplasmic extract with potato acid phosphatase which was prevented by *p*-nitrophenyl phosphate (results not shown) (Figure 6B, lanes 1 and 2). Taken together these results suggest that for the mRNA-binding activity of the 44 kDa protein, free thiol residues and phosphorylation are necessary, either of the protein itself or of some other factors that regulate its activation.

Subcellular localization of the CYP2a5 mRNA 3'-UTR-binding protein

Subcellular fractions were prepared from control and pyrazole-treated mouse livers and tested by UV-cross-linking assays. The

Figure 7 Subcellular localization of the CYP2a5 mRNA 3'-UTR-binding protein

UV-cross-linking assays were performed using different subcellular fractions (see the Experimental section) prepared from control liver (lanes 1, 3, 5 and 7) or pyrazole-treated liver (lanes 2, 4, 6 and 8). Protein (20 μ g) from total cytoplasmic extract (lanes 1 and 2) or cytosol (S130) (lanes 3 and 4) or polyribosomes (lanes 5 and 6) and 2 μ g of protein from nuclear extract (lanes 7 and 8) were used.

Figure 8 Northern-blot analysis of CYP2a5 mRNA

(A) 20 μ g of total RNA (lanes 1 and 2) or polyribosomal RNA (lanes 3 and 4) from control liver (lanes 1 and 3) or pyrazole-treated liver (lanes 2 and 4) were electrophoresed through 1.2% formaldehyde/agarose gel, transferred to nylon membrane and hybridized with CYP2a5 cDNA probe. (B) Ethidium bromide-stained gel controlling RNA loading is shown.

results are summarized in Figure 7 showing that no detectable RNA-binding activity was found in cytosolic fractions (S130) (lanes 3 and 4). Instead, fractions containing polyribosomes showed strong RNA-binding activity that was inducible by pyrazole (lanes 5 and 6). These results demonstrate that the cytoplasmic RNA-binding protein is in fact polyribosomal and are consistent with previous results on proteins that regulate mRNA half-life [38–41]. Interestingly, the RNA-binding activity was also present in nuclear extracts where it appeared to be only weakly inducible compared with the polyribosomal fractions

Figure 9 Effect of phenobarbital on the binding of the 44 kDa protein to CYP2a5 3'-UTR

Cytoplasmic extracts from control (lanes 1, 3 and 5) and phenobarbital-treated (lanes 2, 4 and 6) mice were incubated with probe F, using 2.5 μ g (lanes 1 and 2), 5 μ g (lanes 3 and 4) and 10 μ g (lanes 5 and 6) of protein.

(lanes 7 and 8). Figure 8 shows Northern-blot analysis of CYP2a5 mRNA using either total (lanes 1 and 2) or polyribosomal RNAs (lanes 3 and 4) from control (lanes 1 and 3) or pyrazole-treated mice (lanes 2 and 4). It appears that the polyribosomal CYP2a5 mRNA is greatly increased by pyrazole and that this increase is larger than with total RNA. This result is consistent with the subcellular localization of the CYP2a5 mRNA 3'-UTR-binding activity and could indicate that the RNA-binding protein is involved in the binding and stabilization of the CYP2a5 mRNA to the polyribosomes and/or in the recruitment of the CYP2a5 mRNA into the polyribosomes.

Phenobarbital does not induce the CYP2a5 mRNA 3'-UTR-binding protein

Phenobarbital is a well-known transcriptional activator of several *CYP* genes [42] including *CYP2a5* [13]. Therefore we investigated whether it has any effect on the CYP2a5 3'-UTR-binding protein. UV-cross-linking analyses using cytoplasmic extract from control or phenobarbital-treated mice were performed and the results show that phenobarbital does not stimulate the interaction between the CYP2a5 3'-UTR and the 44 kDa protein; if anything the interaction is decreased (Figure 9). This observation, together with the fact that phenobarbital induces CYP2a5 by a mechanism that does not include mRNA stabilization, is consistent with the hypothesized role of the 44 kDa protein in the stabilization of CYP2a5 mRNA.

Analysis of CYP2a5 mRNA poly(A) tail size

The poly(A) tail plays a major role in the stability of mRNAs [18], and increased stability is often associated with an increased poly(A) tail size [43–47]. Therefore we examined whether, in addition to the RNA–protein interaction described above, the stabilization of CYP2a5 mRNA is associated with an elongation of its poly(A) tail. The poly(A) tail length was determined by comparing the sizes of the mRNA fragments produced by RNase H digestion in the presence of oligonucleotide P4 (see Table 1 and Figure 1) and oligodT with those produced in the presence of P4 but absence of oligodT. The CYP2a5 mRNA hybridizes with the oligonucleotide probes, and addition of the enzyme results in digestion of the RNAs present as RNA–DNA hybrids. When only the P4 is present, the 3'-UTR is cleaved from the remainder of the mRNA, but when both oligonucleotides are present, the poly(A) tail is also removed. Reaction products were

Figure 10 Measurements of CYP2a5 poly(A) tail length

Analysis of poly(A) tail length was performed as described in the Experimental section. Total RNA from control (lanes 1 and 4), phenobarbital-treated (lanes 2 and 5) and pyrazole-treated (lanes 3 and 6) mice were digested with RNase H after incubation with oligonucleotide P4 (see Table 1) either alone (lanes 1–3) or together with oligodT (lanes 4–6).

visualized by hybridizing electrophoretically separated RNAs with a CYP2a5 3'-UTR cRNA probe. Figure 10 shows the results obtained with RNAs extracted from control, phenobarbital- and pyrazole-treated mice. In control and phenobarbital-treated mice, CYP2a5 mRNA has a poly(A) tail containing 30–60 adenines (lanes 1 and 2) whereas CYP2a5 mRNA from pyrazole-treated mice has a poly(A) tail ranging from 30 to 250 adenines (lane 3). When oligodT is added to the incubation mixture, CYP2a5 mRNA fragments from control and treated mice have identical sizes (lanes 4–6). This confirms that the size difference observed in lane 3 compared with lanes 1 and 2 is due to poly(A) tail elongation. These results show that, in addition to the stimulated RNA-protein interactions, CYP2a5 mRNA stabilization by pyrazole is associated with an elongation of the poly(A) tail, and that phenobarbital, which is a transcriptional activator, does not induce the CYP2a5 3'-UTR-binding protein nor increase the poly(A) tail size.

DISCUSSION

In the present study, we show for the first time that mouse liver contains a 44 kDa protein that interacts with a *cis*-element of the 3'-UTR of CYP2a5 mRNA. We also show that, when mice are treated with pyrazole, which enhances hepatic CYP2a5 mRNA levels by stabilization, the size of the poly(A) tails is increased and binding of the 44 kDa protein to the 3'-UTR is stimulated. On the other hand, if mice are treated with phenobarbital, which elevates hepatic mRNA levels through stimulated transcription of the *CYP2a5* gene, no increase in poly(A) tail size or mRNA binding of the 44 kDa protein is observed. These results therefore show that increased binding of the 44 kDa protein to the 3'-UTR of the CYP2a5 mRNA is associated with elongation of the poly(A) tail and with the increased half-life of the mRNA but not with increased transcription and may suggest that the 44 kDa protein has an important role in the stabilization of CYP2a5 mRNA.

Interestingly the 44 kDa protein is found in both nuclei and polyribosomes, and the activity of the polyribosome-bound protein appears to be greatly increased after pyrazole treatment, and is associated with a large increase in polyribosome-bound CYP2a5 mRNA. This is consistent with the subcellular localization of proteins involved in mRNA stabilization and processing and may suggest that the 44 kDa protein is somehow involved in the nuclear and cytoplasmic turnover of the mRNA and/or in the binding and stabilization of the mRNA to the

polyribosomes. For example, similar subcellular localization has previously been shown for AUUUA-binding proteins [35,41,48], and one such protein has been shown to participate in facilitated nucleocytoplasmic transport of mRNA [49]. In yeast, NPL3 protein, which interacts with RNA, shuttles between the nucleus and the cytoplasm and seems to be involved in the nucleocytoplasmic transport of mRNA [50]. The glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase, binds specifically to tRNAs in the nucleus and is suggested to participate in their transport [51]. Furthermore, a 50 kDa protein that interacts with a hairpin structure at the 3' end of histone mRNAs is localized both in the nucleus and polyribosomes and there is evidence suggesting that the protein is involved in the stabilization and transport of the mRNA [52–54].

On the basis of evidence available for other mRNA-binding proteins, the mechanism of stabilization may involve a protective effect of the 44 kDa protein against endonucleolytic cleavage close to or at the site of interaction. This has been proposed for the Xlhbbox 2B mRNA and Xoo 1 mRNA of *Xenopus* [55] and for the transferrin receptor mRNA [56]. Another possible and perhaps more probable mechanism could be that the CYP2a5 3'-UTR-binding protein is involved in the control of poly(A) tail size since CYP2a5 mRNA stabilization by pyrazole is associated with an elongation of the poly(A) tail. Indeed it is known that deadenylation precedes the decay of many eukaryotic mRNAs, and cross-talk(s) between the 3'-UTR sequences of mRNA and the poly(A) tail takes place [57]. On the other hand, instability sequences have been located in the 3'-UTR of mRNAs resulting in a rapid deadenylation [58–61]. One could therefore consider a model in which a protein that interacts with the 3'-UTR of an mRNA inhibits or slows down the rate of deadenylation.

In the nuclei of mammalian cells, the poly(A) tail of mRNA grows to an average length of 200–250 nucleotides [18]. This corresponds to the maximum length of the CYP2a5 mRNA poly(A) tail observed after pyrazole treatment. Shortening of the poly(A) tail is initiated in the cytoplasm [18]. Our results are consistent with a mechanism whereby the 44 kDa protein binds to CYP2a5 mRNA in the nucleus, the ribonucleoprotein complex is then transported to the cytoplasm where the association of the protein with the mRNA maintains a 'long' poly(A) tail by inhibiting its shortening.

The protein-binding site within the CYP2a5 3'-UTR is located between positions 1585 and 1655. Since this site contains one AUUUA motif, its potential role in complex-formation was investigated. UV-cross-linking and competition experiments with an AUUUA-deleted CYP2a5 3'-UTR RNA probe demonstrated that the interaction between the 44 kDa protein and CYP2a5 mRNA is independent of the AUUUA motif. With an RNA probe specifically designed to interact with AUUUA-binding proteins we could, however, demonstrate binding activity with mouse liver cytoplasmic extracts, but this protein has an apparent molecular mass of 40 kDa and is not inducible by pyrazole. We do not know what role this protein may have in mRNA processing, but, on the basis of its molecular mass, it could be similar to proteins that interact with the AU-rich sequences of mRNAs coding for c-Myc [62] or granulocyte-macrophage colony-stimulating factor [63]. We could not demonstrate binding of the 40 kDa protein to the CYP2a5 mRNA. We are not sure why this is, but possibly a single AUUUA motif may not be able to act as an independent *cis*-element since, in most cases, reiterations of AUUUA motifs or an AUUUA motif in the context of an AU-rich sequence is necessary for RNA-protein interaction.

Recently, a few reports have appeared on proteins that interact with mRNA independently of AUUUA motifs and that are

possibly relevant in the control of mRNA stability [39,64–70]. Interestingly, however, the molecular masses of these proteins appear to be different from that of the CYP2a5 mRNA-binding protein. In addition, contrary to other mRNA-binding proteins [38,62,67, 71], the inducible CYP2a5 mRNA-binding protein has no affinity for homopolymeric strands. It seems therefore that the 44 kDa protein described in this paper is different from the other proteins reported previously. Its unique binding characteristics and inducibility by pyrazole also indicate that the interaction with CYP2a5 mRNA is specific.

Biochemical studies show that free thiol and phosphate group(s) on the 44 kDa protein itself or on other regulatory proteins are necessary for the RNA-binding activity. These results could indicate that the protein is up-regulated by post-translational modifications and are in agreement with previous reports on several mRNA-binding proteins [32,37,66,68,69,72].

The molecular mechanisms mediating post-transcriptional regulation of CYP enzymes are still largely unknown. For certain enzymes, stabilization of mRNA has been proposed. These include the increase in rat CYP2B1 and CYP2B2 by dexamethasone [73], rabbit CYP3A6 by triacetyloleandomycin [74], CYP1A1 and CYP1A2 by 2,3,7,8-tetrachlorodibenzodioxin or 3-methylcholanthrene [75–80] and CYP2E1 in diabetic rats [81,82]. It could therefore be that some of these enzymes share the regulation pathway with the CYP2a5 including the 44 kDa protein. This may not be likely, however, for two reasons. First, from our previous studies we know that these enzymes are differently regulated from CYP2a5; for example, exposing mice to pyrazole either reduces their expression or leaves it unchanged [16,83,84]. Secondly, as they belong to other families or subfamilies, their homology is less than 60% with the CYP2a5, and there is no evidence suggesting a highly conserved sequence in the 3'-UTR of their respective mRNAs [1].

On the other hand, there are a few CYP enzymes in the CYP2A subfamily with extremely high homology to CYP2a5 (85–95%) which could share the same regulatory pathway. One of them is rat CYP2A3 which is almost 95% homologous with CYP2a5 [85]. This enzyme is induced by 3-methylcholanthrene in the lung and, interestingly, there seems to be some increase in the mRNA by pyrazole in the nasal epithelium (O. Geneste, F. Raffalli and M. A. Lang, unpublished work). In hamster liver, we have discovered an enzyme similar to mouse CYP2a5, which, like rat CYP2A3, is induced by 3-methylcholanthrene but which, curiously enough, is decreased by pyrazole. We do not know why this is, but it is possible that small differences in the respective mRNA sequences between the hamster and mouse enzymes may lead to differential regulation [86].

In conclusion, it appears that increased expression of CYP enzymes by mRNA stabilization as for CYP2a5 is not a common event within the cytochrome P-450 superfamily and may be limited to only a few members of the subfamily 2A.

In this paper we have provided evidence that a 3'-UTR-binding protein is involved in the stabilization of CYP2a5 mRNA. Although this is the first report demonstrating that such a mechanism may exist for a cytochrome P-450 enzyme, the exact role of the 44 kDa protein in the process is still not understood. For instance, we do not know whether other proteins such as the one that recognizes the AUUUA site are involved. One theoretical possibility for further studies on the role of the 44 kDa protein in the mRNA processing would be to make various recombinants of CYP2a5 mRNA and an appropriate reporter gene to be expressed in a cell line that responds to pyrazole. Unfortunately, no such cell line has been found, but we are trying to find conditions in which hepatocytes will respond to pyrazole or to some other compound or compounds that will

increase the CYP2a5 mRNA and its half-life and the 44 kDa protein in the cells in culture. This may not be straightforward, however, because the increased levels of CYP2a5 mRNA and its increased half-life observed *in vivo* may in fact be the result of a combined effect of an exogenous factor (pyrazole) and endogenous factors not produced in the hepatocytes.

In conclusion, we have shown that if increased levels of CYP2a5 mRNA are achieved in the mouse liver by mRNA stabilization, an elongation of the poly(A) tail as well as increased binding of a 44 kDa protein to the 3'-UTR of CYP2a5 mRNA takes place. If, on the other hand, the mRNA level is increased by stimulated transcription, neither elongation of the poly(A) tail nor stimulated binding of the 44 kDa protein to the 3'-UTR takes place. This suggests that the 44 kDa protein has a role in the stabilization of the CYP2a5 mRNA. The subcellular distribution and biochemical characteristics of the 44 kDa protein are similar to other proteins known to be involved in mRNA stabilization and intracellular transportation of mRNA from the nucleus to polyribosomes. Within the cytochrome P-450 superfamily, this mode of regulation seems to be limited to a few members of the CYP2A subfamily.

Whereas the exact mechanism by which the 44 kDa protein is involved in the stabilization and transportation of the CYP2A5 mRNA is yet to be discovered, its activation by pyrazole is to our knowledge the first example of a mammalian mRNA-binding protein that is activated by a xenobiotic and could therefore be part of a new and so far unknown mechanism by which xenobiotic-metabolizing enzymes respond to environmental stress.

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