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# Heterogeneous Nuclear Ribonucleoprotein A1 and Regulation of the Xenobiotic-Inducible Gene *Cyp2a5*

FRANÇOISE RAFFALLI-MATHIEU, TINA GLISOVIC, YAACOV BEN-DAVID, and MATTI A. LANG

*Division of Biochemistry, Department of Pharmaceutical Biosciences, Faculty of Pharmacy, Biomedical Centrum, University of Uppsala, Sweden (F.R.-M., T.G., M.A.L.); and Department of Medical Biophysics, University of Toronto, Sunnybrook and Women's College Health Science Centre, Toronto, Ontario, Canada (Y.B.-D.).*

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## ABSTRACT

The heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) functions in the packaging of nascent RNA polymerase II transcripts and participates in a variety of nuclear and cytoplasmic processes that modulate gene expression. The RNA binding characteristics of hnRNP A1 suggest that it can modulate the expression of specific genes, but little is known about its possible targets in vivo. In this article, we show that hnRNP A1 interacts with the transcript of a cytochrome P450 gene, *Cyp2a5*, induced by xenobiotics and during liver damage. Binding of the hnRNP A1 to CYP2A5 mRNA was demonstrated by immunoprecipitation of the xenobiotic-stimulated (37/39 kDa) CYP2A5 mRNA-protein complex with a monoclonal anti-hnRNP A1 antibody, by partial trypsin digestion of the complex, and by showing that the RNA-protein complex is not formed with protein extracts from cells lacking the hnRNP A1. We also show that a specific hepatotoxic inducer of the *Cyp2a5* gene, pyrazole, increases the cytoplasmic levels of hnRNP A1 in vivo. Finally, we show that hnRNP A1 can be overexpressed in mouse primary hepatocytes, leading to an accumulation of the CYP2A5 mRNA. Collectively, these results indicate that the hnRNP A1 is an important regulator of the *Cyp2a5* gene.

Cytochromes P450 (P450s) form a large and diverse superfamily of genes coding for enzymes involved in the oxidative metabolism of various xenobiotics and endogenous molecules (Nelson et al., 1996; Rendic and Di Carlo, 1997). The expression of many P450 genes is modulated by xenobiotics, and the regulation can take place at different levels ranging from transcriptional activation to protein stabilization (Porter and Coon, 1991). The transcriptional activation of P450 genes has been widely studied; recently, a number of xenobiotic-activated receptors that act as specific transcription factors have been discovered (Waxman, 1999). Other than transcription, post-transcriptional control is important in the regulation of several P450s (Simmons et al., 1987; Silver and Krauter, 1990; Peng and Coon, 1998); however, the molecular mechanisms involved are poorly known.

We used the *Cyp2a5* gene as a model to understand the molecular mechanisms of gene induction by xenobiotics. The gene product activates the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (Felicia et al., 2000) and is believed to play an important role in liver carcinogenesis (Wastl et al., 1998). The *Cyp2a5* gene is up-regulated by many compounds and agents causing liver injury (Kirby et al., 1994; Camus-Randon et al., 1996) and in certain liver tumors (Wastl et al., 1998), yet the mechanism of induction is not known. Some xenobiotics, such as the classic P450 inducer phenobarbital, up-regulate *Cyp2a5* transcriptionally, whereas others, such as pyrazole, induce it by increasing the stability of the mRNA (Aida and Negishi, 1991; Hahnemann et al., 1992). When examining the molecular mechanisms of CYP2A5 mRNA stabilization, we discovered a protein with an apparent molecular mass of 37/39 kDa that binds to the 3′-untranslated region (UTR) of the CYP2A5 mRNA. The mRNA/protein complex formation is stimulated by pyrazole but not by phenobarbital. We also found that pyrazole causes an elongation of the CYP2A5 mRNA poly(A) tail (Geneste et al., 1996), indicating that the 37/39-kDa protein could regulate the CYP2A5 mRNA stability by controlling the length of the transcript's poly(A) tail. To our knowledge, the 37/39-kDa protein is the first transacting factor identified that may regulate the stability of a drug-induced P450 mRNA.

In this article, we establish that the 37/39-kDa protein that binds to the CYP2A5 mRNA is the heterogeneous nuclear ribonucleoprotein (hnRNP) A1. Moreover, we show that the amount of cytoplasmic hnRNP A1 is increased by pyrazole treatment in vivo, and we present evidence indicating that it participates in the regulation of the expression of the *Cyp2a5* gene. This study sheds light on the molecular mechanism of

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**ABBREVIATIONS:** P450, cytochrome P450; UTR, untranslated region; hnRNP, heterogeneous nuclear ribonucleoprotein; nt, nucleotide(s); kb, kilobase(s); PAGE, polyacrylamide gel electrophoresis; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase.

*Cyp2a5* gene regulation and demonstrates that hnRNP A1 activity can be regulated by a toxic xenobiotic.

## Materials and Methods

**Animals.** Male DBA/2J mice aged 6 to 8 weeks were obtained from Møllegaard (Ejby, Denmark). The mice were treated with i.p. injections of pyrazole (180 mg/kg) or phenobarbital (80 mg/kg) dissolved in saline, or with saline only, for 3 consecutive days. The mice were killed 24 h after the last injection, and the livers were removed. The studies were approved by the Ethical Committee (Uppsala, Sweden; approval number C3/1) and were performed accordingly.

**Cells.** Mouse primary hepatocytes were prepared from untreated mice according to a two-step perfusion model described previously (Seglen, 1972). The isolated hepatocytes were dispersed in Williams' E medium containing 20 ng/ml dexamethasone, insulin/transferrin/sodium selenite (5 mg/l insulin, 5 mg/l transferrin, 5 $\mu$g/l sodium selenite), 10 $\mu$g/ml gentamicin, 1% L-glutamine, and 10% decomplemented fetal calf serum at a density of $1.8 \times 10^6$ cells/60-mm uncoated culture dish (Corning, Palo Alto, CA). The cells were maintained at 37°C in 5% $CO_2$ in a humidified incubator. After 2 h of incubation, the medium was changed to Williams' E medium without fetal calf serum. The mouse erythroleukemia cell line CB3 lacking hnRNP A1 and its derivative re-expressing this gene have been described elsewhere (Yang et al., 1994). They were cultured in minimal essential medium $\alpha$ medium supplemented with 10% fetal calf serum and 800 $\mu$g/ml geneticin.

**Transfection of the pCG-hnRNP A1 Plasmid in Mouse Primary Hepatocytes.** The primary hepatocytes were cultured for 24 h in Williams' E medium before transfection. Cells were transfected using LipofectAMINE PLUS (Invitrogen AB, Täby, Sweden) with 8 $\mu$g of the pCG-A1 containing the hnRNP A1 cDNA (kindly provided by Dr. Adrian Krainer, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) or 8 $\mu$g of the pCG plasmid (obtained by removing the hnRNP A1 cDNA sequence inserted at the *Bam*HI and *Xba*I sites of the pCG plasmid). Cotransfection with the $\beta$-galactosidase–expressing plasmid pCMV-SPORT-$\beta$Gal (Invitrogen) was performed to estimate the transfection efficiency.

**Isolation of Nuclear and Cytoplasmic Proteins.** Crude cytoplasmic extracts from mouse liver were prepared as described previously (Geneste et al., 1996). Mouse primary hepatocytes and erythroleukemia cells were washed and resuspended in phosphate-buffered saline. The cell suspension was centrifuged at 2000*g* for 30 s, and the pellet was resuspended in buffer A [10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl-fluoride, 10 $\mu$g/ml leupeptine, and 0.4% Igepal (Aventis, Strasbourg, France)] and kept on ice for 60 min. The cell suspension was vortexed, homogenized, and centrifuged at 11,000*g* at 4°C for 10 min. The supernatant containing the cytoplasmic proteins was stored at −80°C. The pellet containing the nuclei was resuspended in buffer B (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl-fluoride, and 0.4% Igepal) and gently agitated for 30 min at 4°C. The suspension was centrifuged at 11,000*g* for 15 min at 4°C. The supernatant containing the nuclear proteins was stored at −80°C. Protein content was measured by use of the Lowry method (Lowry et al., 1951).

**Preparation of Radiolabeled RNA.** The RNA probe was synthesized by in vitro transcription of CYP2A5 cDNA amplified by polymerase chain reaction in the presence of [$\alpha$-$^{32}$P]UTP, as described previously (Geneste et al., 1996). The RNA probe used in all UV crosslinking experiments is 71 nt long and contains the primary binding site of the 37/39-kDa protein. This probe has been described elsewhere (Geneste et al., 1996; Tilloy-Ellul et al., 1999).

**UV Crosslinking.** UV crosslinking was performed as described previously (Geneste et al., 1996). Briefly, 10 $\mu$g of cytoplasmic lysates or 5 $\mu$g of nuclear proteins was incubated with $2 \times 10^5$ cpm of radiolabeled 71-nt CYP2A5 RNA. The mixture was subjected to UV crosslinking and was treated with RNase A. Proteins were denatured and separated using SDS-PAGE, and the gel was dried and autoradiographed.

**Partial Proteolysis of the RNA/Protein Complex.** For partial proteolysis experiments, the conditions described by Hamilton et al. (1993) were used. In short, UV crosslinking was performed with cytoplasmic lysates as described above, and immediately after digestion with RNase A, 50, 100, or 500 ng of trypsin (Roche Applied Science, Stockholm, Sweden) was added to the mixtures for the indicated times. The samples were then denatured and analyzed by SDS-PAGE and autoradiography.

**Immunoprecipitation of the RNA/Protein Complexes.** Immunoprecipitation was performed essentially as described by Hamilton et al. (1993). The CYP2A5 RNA/protein complexes (15-$\mu$g cytoplasmic proteins) were incubated with 1:500 dilution of the indicated antibody (kindly provided by Dr. Gideon Dreyfuss, Howard Hughes Medical Institute, University of Philadelphia, Philadelphia, PA) and then immunoprecipitated with protein A-Sepharose beads (Pharmacia AB, Uppsala, Sweden). After washing, the pelleted complexes were denatured and separated by SDS-PAGE. Radioactive complexes were detected by the use of autoradiography.

**Western Blot Analysis.** Western blots were performed using 50 $\mu$g of cytoplasmic and nuclear proteins, as described previously (van der Houven van Oordt et al., 2000), with 1:1000 dilution of the 9H10 (anti-hnRNP A1) antibody.

**RNA Isolation and Northern Blot Analysis.** Total cellular RNA was isolated from primary mouse hepatocytes using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). Total RNA (10 $\mu$g) was size-fractionated on a 1.2% agarose/formaldehyde gel and transferred to a Hybond-N nylon membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). The CYP2A5 cDNA (provided by Dr. Masahiko Negishi, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC) was radiolabeled with [$\alpha$-$^{32}$P]dCTP using the Megaprime labeling kit (Amersham). Hybridization was performed with $1.7 \times 10^7$ cpm of radiolabeled probe at 65°C overnight in Church buffer (Church and Gilbert, 1984) modified to contain 0.25 M phosphate buffer, 7% SDS, and 1 mM EDTA. The filter was washed $2 \times 5$ min at room temperature in a buffer containing 2× SSC and 0.1% SDS and then $1 \times 15$ min at 65°C in a buffer containing 2× SSC and 1% SDS. To assess equal loading of the samples, the mRNA level of the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was measured using the GAPDH cDNA (CLONTECH, Palo Alto, CA) as a probe.

For the densitometric analysis, scanning of the film was performed with a Scanjet 4c scanner (Hewlett Packard, Palo Alto, CA), and quantification was conducted using the software NIH Image 1.61 (http://rsb.info.nih.gov/nih-image/).

## Results and Discussion

**Identification of the 37/39-kDa CYP2A5 mRNA Binding Protein as hnRNP A1.** In previous studies, we identified in mouse liver a protein with an apparent molecular mass of 37/39 kDa binding to a putative hairpin-loop structure at the 3′UTR of the CYP2A5 mRNA. We also showed that the *Cyp2a5*-specific inducer pyrazole stimulates the interaction of the protein with CYP2A5 mRNA. Because certain characteristics of the 37/39-kDa protein suggest that it could be one of the hnRNPs (Geneste et al., 1996; Tilloy-Ellul et al., 1999), a set of experiments was designed to establish the identity of this protein. Figure 1 shows that the pyrazole-activated 37/39-kDa RNA/protein complex can be immunoprecipitated by a monoclonal antibody against the hnRNP A1 (9H10) but not by an antibody against the hnRNP C (4F4).

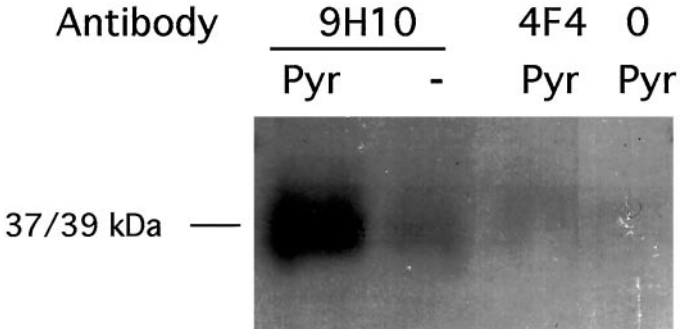


**Fig. 1.** SDS-PAGE analysis of immunoprecipitated RNA/protein complexes. UV crosslinking was performed on liver cytoplasmic extracts from pyrazole-treated (Pyr) and untreated (−) mice (see *Materials and Methods*) incubated with a 71-nt radioactive RNA probe corresponding to the 37/39-kDa protein binding site in CYP2A5 3′UTR. The RNA-protein complexes were immunoprecipitated using the 9H10 (anti-hnRNP A1) antibody, the 4F4 (anti-hnRNP C) antibody, or no antibody (0). The immunoprecipitated complex was analyzed by SDS-PAGE. The 37/39-kDa complex is indicated.

Partial proteolytic cleavage of the complex was then carried out using trypsin. This treatment has been shown previously to convert the hnRNP A1 into a 25-kDa fragment retaining RNA binding activity (Hamilton et al., 1993). The protein extract containing the 37/39-kDa protein irreversibly bound to the radioactive CYP2A5 RNA probe by UV crosslinking was incubated with trypsin, as described under *Materials and Methods*. Figure 2 shows that the characteristic 25-kDa complex is obtained. The simultaneous disappearance of the 37/39-kDa complex and appearance of a 25-kDa species indicates that the 25-kDa complex derives primarily from the 37/39-kDa complex. Finally, we verified that the 37/39-kDa complex consists of the hnRNP A1 by comparing the complex formation using protein extracts prepared from two mouse erythroleukemia cell lines that differ in their ability to express the hnRNP A1. The CB3 cells have a retroviral insertion near the hnRNP A1 gene, encoding the hnRNP A1 and have lost the second hnRNP A1 allele (Ben-David et al., 1992). Consequently, they do not produce detectable hnRNP A1 protein. The CB3+ cells have been stably infected with a retrovirus containing the murine hnRNP A1 coding sequence and the CB3− cells with the empty vector (Yang et al., 1994). Figure 3 shows that no 37/39-kDa complex is formed using cytoplasmic and nuclear extracts from the CB3− cells, whereas the 37/39-kDa complex is readily formed with extracts from the CB3+ cells. These three different approaches allowed us to conclude that the hnRNP A1 is the major protein interacting with CYP2A5 mRNA 3′UTR in a xenobiotic-regulated manner.

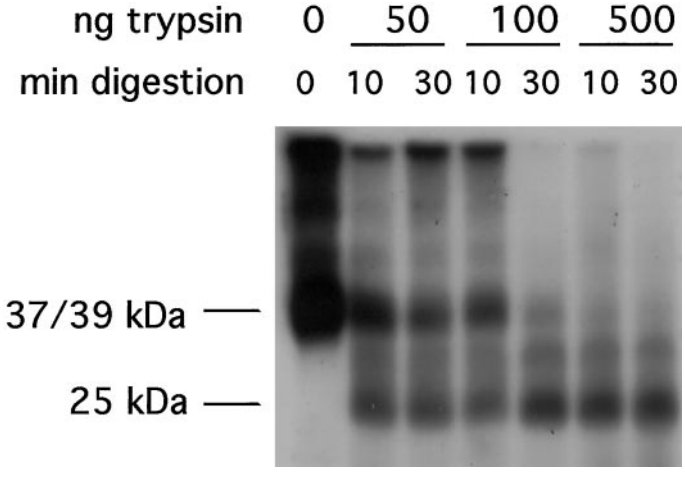


**Fig. 2.** Partial proteolysis of the 37/39 kDa complex. UV crosslinking was performed with liver cytoplasmic extracts from pyrazole-treated mice and the radioactive CYP2A5 RNA probe. RNA-protein complexes were incubated in the presence of 50, 100, or 500 ng of trypsin for 10 or 30 min and were analyzed by SDS-PAGE. Molecular masses of the complexes are indicated.

**Pyrazole Increases the Cytoplasmic Level of hnRNP A1.** Having established that the protein present in the 37/39-kDa complex is the hnRNP A1, we investigated whether pyrazole affects its level in mouse liver. To this end, we performed a Western blot analysis using cytoplasmic and nuclear protein extracts from the liver of untreated or pyrazole-treated mice. For comparison, we also used another P450 inducer, phenobarbital, known to induce *Cyp2a5* by a different mechanism than pyrazole (Hahnemann et al., 1992). Figure 4 shows that two bands are detected. Because the antibody used also recognizes the minor splice variant hnRNP $A1^B$, we conclude that the bands correspond to the hnRNP A1 (34 kDa) and the hnRNP $A1^B$ (37 kDa) (Ben-

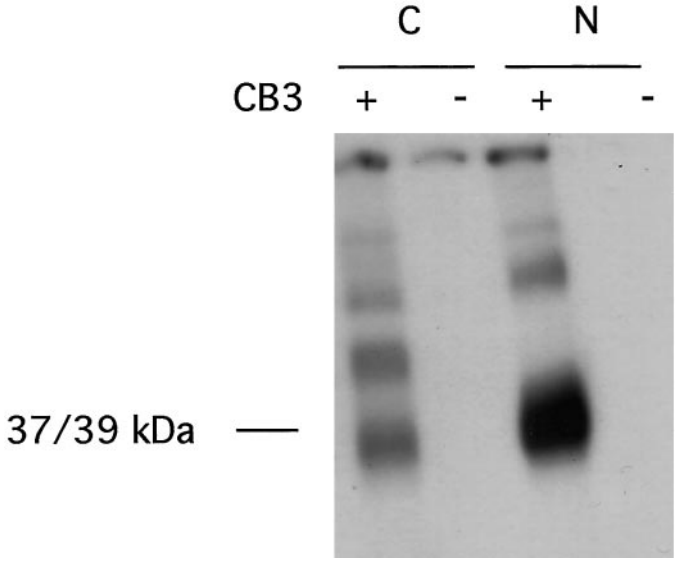


**Fig. 3.** Formation of the RNA/protein complexes in CB3 cells. UV crosslinking was performed using 10 μg of cytoplasmic proteins (C) or 5 μg of nuclear (N) proteins prepared from CB3+ and CB3− cells and the CYP2A5 RNA probe. The 37/39-kDa complex is indicated.

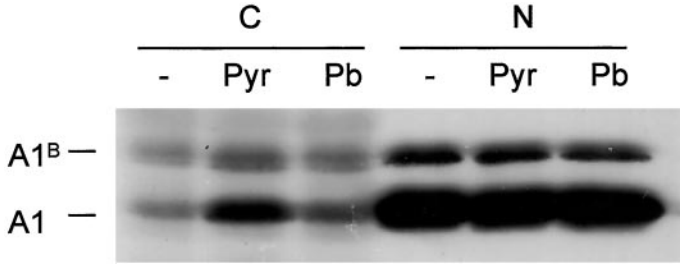


**Fig. 4.** hnRNP A1 levels in mice treated with pyrazole and phenobarbital. Western blot of crude protein extracts (C) and nuclear (N) proteins (50 μg) prepared from untreated (−), pyrazole (Pyr)-treated, and phenobarbital (Pb)-treated mice was performed. The 9H10 antibody was used to detect the hnRNP A1. A band at 34 kDa, corresponding to the hnRNP A1, was detected. The band at 37 kDa most probably represents the hnRNP $A1^B$ splice variant.

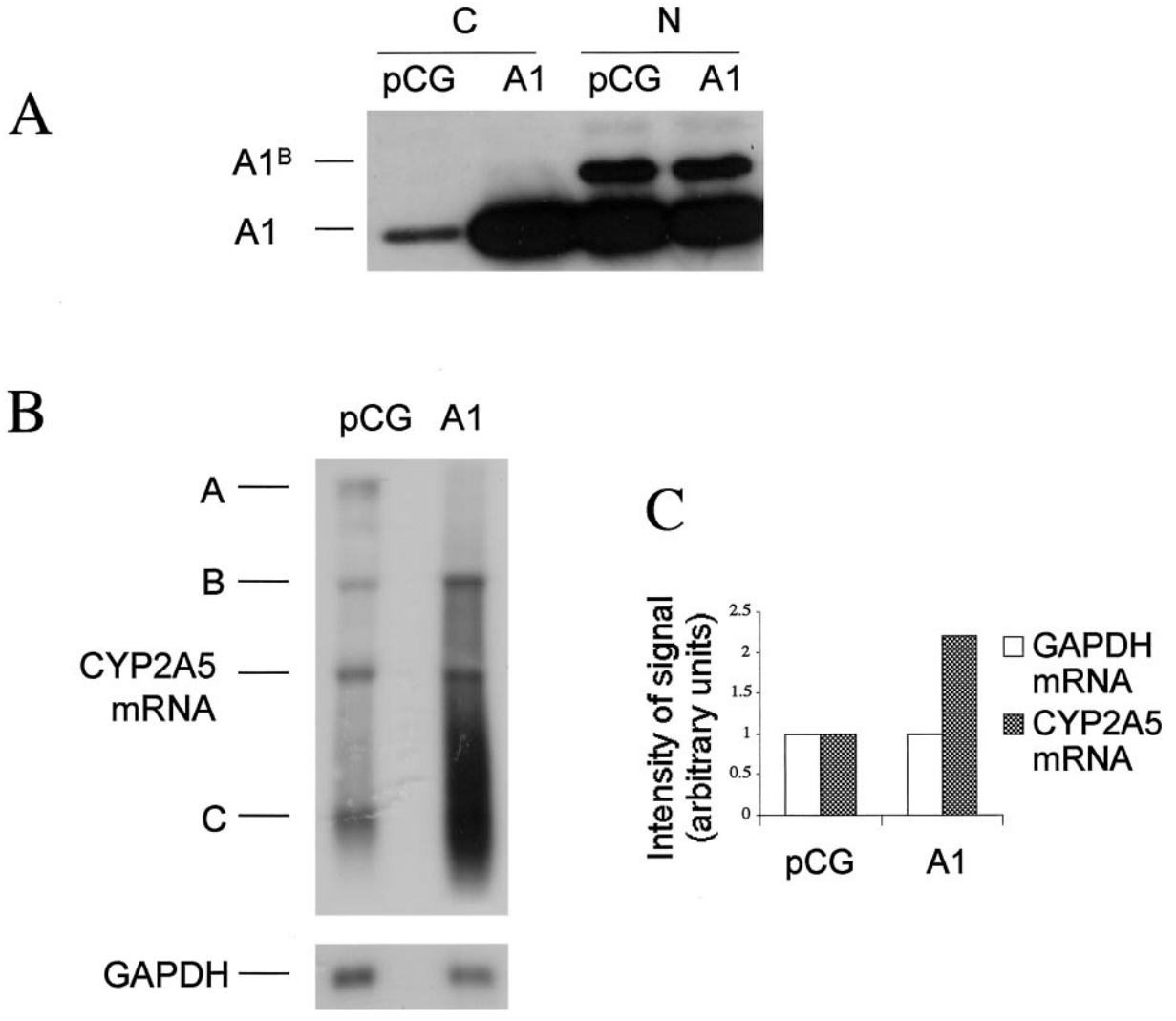


**Fig. 5.** Overexpression of hnRNP A1 in mouse primary hepatocytes. A, efficacy of overexpression. Western blot analysis was performed with cytoplasmic (C) and nuclear (N) protein extracts (15 $\mu$g) prepared 24 h after transfection of the primary hepatocytes with the pCG-hnRNP A1 (A1) or pCG alone (pCG) (not containing the hnRNP A1 cDNA, see *Materials and Methods*). The 9H10 antibody was used to detect the hnRNP A1. B, Northern blot analysis of RNA (10 $\mu$g) isolated from mouse primary hepatocytes transfected with the pCG-A1 or pCG plasmids. Top, a CYP2A5 cDNA probe was used. The main bands detected are indicated (A, 11 kb; B, 3.8 kb; CYP2A5 mRNA, 1.9 kb; C, 400 nt). Bottom, a GAPDH cDNA probe was used to assess for equal RNA loading. C, quantification of the CYP2A5 mRNA amounts in cells transfected with the pCG or pCG-A1 (A1) plasmids. The intensity of the band corresponding to the CYP2A5 mRNA relative to the GAPDH mRNA is shown.

David et al., 1992; LaBranche et al., 1998). Whereas phenobarbital does not seem to affect hnRNP A1 levels significantly, pyrazole causes a large increase of cytoplasmic hnRNP A1. Because the A1 protein is predominantly nuclear, we cannot conclude from our results whether the increase in cytoplasmic hnRNP A1 from pyrazole addition is caused by a translocation of the hnRNP A1 from the nucleus to the cytoplasm or by increased hnRNP A1 protein synthesis. However, these and previous results (Geneste et al., 1996) indicate that the increase in cytoplasmic CYP2A5 RNA/hnRNP A1 complex formation can be explained by an increased level of A1 protein and that this could be important for the regulation of CYP2A5 mRNA stability. The situation is different in the nucleus, where increased complex formation (Geneste et al., 1996) is not accompanied by an increase in A1 protein levels. This implies that the mechanisms of activation of the nuclear and cytoplasmic A1 proteins by pyrazole are at least partially distinct. Others have reported that nuclear and cytoplasmic hnRNP A1 can react differently to a given stimulus. For example, actinomycin D differently regulates the RNA-binding activity of nuclear and cytoplasmic hnRNP A1 (Hamilton et al., 1997). More work is needed to completely understand how pyrazole increases the cytoplasmic level of hnRNP A1. Many stimuli known to impair RNA polymerase II activity cause a relocalization of the hnRNP A1 to the cytoplasm (Pinol-Roma and Dreyfuss, 1992; Vautier et al., 2001). Epidermal growth factor and retinol have been shown to induce the hnRNP A1 gene (Planck et al., 1988; An and Wu, 1993). In preliminary experiments, we observed that pyrazole treatment does not affect the hnRNP A1 mRNA level in mouse liver (data not shown), suggesting that pyrazole could affect hnRNP A1 subcellular localization or translation.

**Overexpression of hnRNP A1 in Primary Hepatocytes Induces the *Cyp2a5* Gene.** We wanted to gain insight into the possible functions of hnRNP A1 in the regulation of the *Cyp2a5* gene expression. For that purpose, the hnRNP A1 was overexpressed in mouse primary hepatocytes

by transfecting the cells with a plasmid expressing the hnRNP A1 (pCG-A1). Cells transfected with the same plasmid lacking the hnRNP A1 cDNA (pCG) were used as control cells. Figure 5A shows that the level of cytoplasmic hnRNP A1 strongly increases 24 h after transfection of the pCG-A1, compared with cells transfected with the pCG plasmid. Using a CYP2A5 cDNA probe, we then analyzed by Northern blot whether CYP2A5 mRNA levels were affected by hnRNP A1 overexpression. Figure 5B shows that the CYP2A5 mRNA (1.9 kb) and three other RNA species are detected by the radiolabeled probe: A (approximately 11 kb), B (approximately 3.8 kb), and C (approximately 400 nt). The A, B, and C RNA species were not characterized further. However, from their size, we can hypothesize that the 3.8-kb RNA corresponds to a CYP2A5 pre-mRNA, and the 400-nt RNA corresponds to a degradation product of the mature CYP2A5 transcript.

A densitometric analysis of the CYP2A5 mRNA band shows that its intensity is increased by a factor of 2.2 in cells overexpressing the hnRNP A1 (Fig. 5C). Together with our previous results, this strongly suggests that the hnRNP A1 regulates the level of CYP2A5 transcript. This is supported by our preliminary experiments indicating that the hnRNP A1 primary binding site on CYP2A5 3′UTR functions as an hnRNP A1-dependent stability determinant of the CYP2A5 mRNA (data not shown).

In conclusion, we show in this article that the hnRNP A1 binds to the 3′UTR of the CYP2A5 mRNA and is likely to be a key regulatory factor for *Cyp2a5* gene expression. Other studies have shown that hnRNP A1 participates in alternative splicing (Yang et al., 1994) and mRNA export (Nakielny and Dreyfuss, 1997), and it may affect mRNA stability (Hamilton et al., 1993; Blaxall et al., 2000) and translation (Svitkin et al., 1996). Studies aimed at understanding the precise mechanisms of the regulatory action of hnRNP A1 on *Cyp2a5* expression, in particular its possible involvement in pre-mRNA processing, mRNA turnover, and translation, are ongoing in our laboratory.

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**Address correspondence to:** Dr. Françoise Raffalli-Mathieu, Division of Biochemistry, Department of Pharmaceutical Biosciences, Faculty of Pharmacy, Box 578 Biomedicum, SE-751 23 Uppsala, Sweden. E-mail: francoise.mathieu@farmbio.uu.se