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Interplay between transcriptional and post-transcriptional regulation of *Cyp2a5* expression

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Abstract

The cytochrome P450 (*Cyp*) *2a5* gene can be upregulated transcriptionally or by mRNA stabilization. The heterogeneous nuclear ribonucleoprotein (hnRNP) A1 interacting with the CYP2A5 mRNA has been shown to be a key post-transcriptional regulator of the *Cyp2a5* gene. The aim of this study was to investigate if the transcriptional and post-transcriptional steps of *Cyp2a5* expression are linked. This was done by modifying the transcription rate with transcriptional inducers (phenobarbital and cyclic AMP) and inhibitors (actinomycin D and 5,6-dichloro-1-beta-d-ribofuranosylbenzimidazole) and analyzing the effects upon post-transcriptional events. We found that inhibition of transcription led to relocalization of hnRNP A1 from the nucleus to the cytoplasm, to its strongly increased binding to the cytoplasmic CYP2A5 mRNA and to CYP2A5 mRNA stabilization. In contrast, stimulated transcription resulted in increased binding of nuclear hnRNP A1 to the *Cyp2a5* promoter, and overexpression of hnRNP A1 led to stimulated transcription of a *Cyp2a5* promoter-driven luciferase recombinant. This strongly suggests that the transcriptional and post-transcriptional stages of *Cyp2a5* expression are interrelated and that the nucleocytoplasmic shuttling hnRNP A1 may coordinate these different steps.

Keywords: hnRNP A1; *Cyp2a5*; Transcription; mRNA stability; Interplay; RNA–protein interaction

1. Introduction

Increasing evidences indicate that links exist between transcriptional and post-transcriptional steps of gene expression, allowing integration and guaranteeing an efficient control of this multistep process. It has been suggested that the integration is achieved by multifunctional proteins playing a role in several stages along the expression pathway [1,2]. Cytochrome P450 (*Cyp*) genes encode for a superfamily of haemoproteins involved in the oxidation of many exogenous and endogenous molecules [3,4]. Xenobiotic-mediated induction of some of these genes, a means for organisms to adapt to chemical stress, is achieved through both transcriptional and post-transcriptional mechanisms [5]. Recent identification of drug-activated transcription factors has shed light on the molecular events of *Cyp* gene transcriptional regulation [6], but much less is known about their post-transcriptional control and its possible coupling to transcription.

The CYP2A5 enzyme catalyzes the metabolism of several toxins and its human orthologue is known as the nicotine hydroxylase [7]. The *Cyp2a5* gene is inducible by several structurally diverse toxic xenobiotics [8], and its expression can be regulated both transcriptionally and post-transcriptionally. For example, phenobarbital (PB) stimulates its transcription [9], whereas pyrazole increases the CYP2A5 mRNA half-life [10]. The *Cyp2a5* gene therefore serves as a good model for studies on the integration of different steps in xenobiotic-mediated gene induction.

We have shown that hnRNP A1, a multifunctional protein involved in various stages of mRNA metabolism [11,12], is a key post-transcriptional regulator of the *Cyp2a5* gene [13–15]. It binds to the CYP2A5 mRNA 3′UTR, and it is involved in the stabilization of the transcript, most likely by controlling the length of its poly(A) tail.

The aim of the present study was to investigate whether the post-transcriptional regulation of the *Cyp2a5* gene is

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Abbreviations: Cyp, cytochrome P450; PB, phenobarbital; hnRNP, heterogeneous nuclear ribonucleoprotein; UTR, untranslated region; ActD, actinomycin D; DRB, 5,6-dichloro-1-beta-d-ribofuranosylbenzimidazole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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linked to its transcriptional control. For that purpose, we analyzed how modification of transcriptional activity may influence post-transcriptional events of *Cyp2a5* expression. In cultured mouse primary hepatocytes, we found that when transcription is inhibited, hnRNP A1 and its associated CYP2A5 mRNA binding activity are relocalized from the nucleus to the cytoplasm, and CYP2A5 mRNA is stabilized. However, during basal or stimulated transcriptional activity, hnRNP A1 stays in the nucleus, interacts with the *Cyp2a5* promoter, and seems to play a role in the transcription of the *Cyp2a5* gene. The results propose that the transcriptional and post-transcriptional events in the expression of the *Cyp2a5* gene are coordinated by the multifunctional protein hnRNP A1, playing a role in both the nuclear and cytoplasmic events. The function of hnRNP A1 as a coordinator of the expression of the drug inducible *Cyp2a5* gene, as well as the purpose of this type of regulation, are discussed.

2. Materials and methods

2.1. Animals

Male DBA/2J mice, aged 6–9 weeks, were provided by Möllegaard. They were allowed to acclimatize for 1 week before use in hepatocyte isolation. During this time, the mice were kept at the animal facility at BMC, Uppsala, and fed chow and water *ad libitum*. The studies were approved by the Ethical Committee (Uppsala, Sweden; approval number C3/1) and were performed accordingly.

2.2. Isolation and treatment of primary mouse hepatocytes

Isolation of hepatocytes was performed according to a two-step perfusion model as previously described [16]. The isolated hepatocytes were dispersed in Williams' medium E containing dexamethasone (20 ng/mL), ITS (insulin 5 mg/L, transferrin 5 μg/L, sodium selenite 5 mg/L), gentamicin (10 μg/mL), 1% L-glutamine and 10% decomplemented fetal calf serum at a density of 5×10^6 cells/100-mm uncoated culture dish (Corning). The cells were maintained at 37°, 5% CO_2 in a humidified incubator. After 2 hr of incubation, the medium was changed to Williams' medium E without fetal calf serum. Treatment of the cells was initiated 24 hr after plating. ActD and DRB were dissolved in DMSO, whereas cAMP and PB were dissolved in culture medium.

2.3. RNA isolation and Northern blot analysis

Total cellular RNA was isolated from primary mouse hepatocytes and mouse liver using RNeasy Mini and Midi kits (QIAGEN) according to the manufacturer's protocol. 10–20 μg total RNA was size-fractionated by electrophoresis through a 1.2% agarose/formaldehyde gel, transferred to a Hybond^{TM}-N nylon membrane (Amersham Biosciences) and UV crosslinked before hybridization. For Northern analysis, plasmids containing the full length CYP2A5 cDNA (kindly provided by M. Negishi, NIEHS), and the CYP2B10 cDNA (a gift from P. Honkakoski, University of Kuopio) were radiolabeled with [α-^{32}P]dCTP mersham Biosciences). Hybridization was performed with 1.7×10^7 cpm of radiolabeled probe at 42° overnight. In order to assess equal loading of the samples, the mRNA level of the housekeeping gene GAPDH was measured using radiolabeled GAPDH cDNA (CLONTECH).

2.4. Poly(A) tail analysis

The extent of adenylation of CYP2A5 mRNA was estimated using the method described by Brewer and Ross [17], with some modifications. Total RNA (30 μg) from untreated or ActD-treated hepatocytes, was incubated with 500 ng of the antisense oligonucleotide 5′-TGTAGGTTGGTGGGATCGTG-3′, corresponding to nucleotides 1443–1462 in CYP2A5 cDNA [13]. The DNA/RNA hybrid was digested using 1.2 units of RNase H (Life Technologies) at 37° for 1 hr. The digestion releases two types of RNA fragments: the 5′ portion of the transcript, upstream of the 1443–1462 region, and the 3′ part of the transcript containing the 227 nucleotides downtream of the 1443–1462 region plus the poly(A) tail. In parallel digestion reactions, oligo dT was added to allow RNase H cleavage of the poly(A) tails. The digested samples were electrophoresed through a 2.5% low melting point agarose/formaldehyde gel, blotted on to a Hybond^{TM}-N nylon filter (Amersham Biosciences) and fixed under UV light. An *in vitro* transcribed [α-^{32}P]UTP-labeled cRNA probe complementary to nucleotides 1456–1689 in the CYP2A5 mRNA, was used to detect the adenylated and deadenylated 3′ fragments released by the RNase H.

2.5. Isolation of nuclear and cytoplasmic proteins

Hepatocytes were harvested in PBS using a rubber policeman. 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 DTT, 0.2 mM PMSF, 10 μg/mL leupeptine, 0.4% Igepal) and left on ice for 1 hr. After homogenization, the cells were centrifuged at 11 000 *g*, 4° for 10 min. The supernatant containing the cytoplasmic proteins was stored at −80°. The pellet containing the nuclei was resuspended in cold 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 DTT, 0.5 mM PMSF, 0.4% Igepal) and stirred with a magnetic stirrer for 30 min at 4°. The suspension was homogenized and centrifuged at 11 000 *g* for 15 min at 4°. The supernatant containing the nuclear proteins was stored at −80°.

2.6. UV crosslinking assays

A 71 nt RNA probe containing the hnRNP A1 protein binding site present in the CYP2A5 3′UTR was prepared and binding reactions were performed as described in Geneste *et al*. [13]. Nuclear (5 μg) and cytoplasmic (10 μg) proteins were used. The samples were exposed to UV light for 20 min in a Spectrolinker XL-1000 UV-crosslinker (Spectronics). Unprotected RNA was digested with 2 μg of RNase A (Life Technologies) at 37° for 20 min. The samples were denatured under non-reducing conditions and proteins were separated by SDS/PAGE (12%). Finally, the gel was dried and autoradiographed overnight.

2.7. Western blot

Proteins (50 μg) were isolated as described above and separated by SDS/PAGE (12%) and blotted onto a nitrocellulose membrane, Hybond^{TM}-C extra (Amersham Biosciences). A monoclonal anti-hnRNP A1 antibody, 9H10, kindly provided by G. Dreyfuss (Howard Hughes Medical Institute, University of Philadelphia) was used in a 1:1000 dilution. All solutions contained 5% non-fat dry-milk. Finally, detection was performed with the ECL Western blotting analysis system (Amersham Biosciences).

2.8. Transfection of primary mouse hepatocytes

Primary mouse hepatocytes were plated at a density of 1.8×10^6 cells/60-mm uncoated culture dish (Corning) and cultured for 24 hr in Williams' medium E before transfection. The cells were transfected using Lipofectamine PLUS (Invitrogen) with 1 μg of either the *Cyp2a5* promoter-driven pGL3 Basic luciferase plasmid (kindly provided by J. Hakkola, University of Oulu) or an SV40 promoter-driven luciferase plasmid (pGL3 control) from Promega, and 2 μg of the pCG-A1 plasmid containing the hnRNP A1 cDNA (a kind gift from A. Krainer, Cold-Spring Harbor Laboratory), or 2 μg 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 a β-galactosidase-expressing plasmid, the pCMV-SPORT-βGal (Invitrogen), was performed to estimate the transfection efficiency.

2.9. Luciferase assay

The hepatocytes were harvested 48 hr after transfection. All reagents needed for the luciferase assay were from Promega. In short, the cells were washed with cold PBS and lysed in reporter lysis buffer (RLB). One freeze–thaw cycle was performed on the cell lysates. Before assaying the luciferase activity, the lysates were centrifuged at 9500 *g* for 2 min. 30 μL of the supernatant were added to 100 μL luciferase assay reagent, and the luminescence in relative light units (RLU) was read in a TD-20/20 luminometer (Turner Designs). The β-galactosidase activity was determined for each lysate as described by Rosenthal [18], and the RLU values were normalized to the β-galactosidase activities.

2.10. Oligonucleotides

Double-stranded DNA corresponding to either the −543 to −272 region (probe 1) or the −282 to −4 region (probe 2) in the *Cyp2a5* promoter were generated by PCR. Primers 1 and 2 were used to amplify probe 1, whereas primers 3 and 4 were used to amplify probe 2.

The sequences of the primers are: 5′-CTCTTCATTGAAAGACTCCC-3′ (primer 1); 5′-GACACTTCCTCTGAGAAGGG-3′ (primer 2); 5′-TGATGGCTGAATTAGCTTGC-3′ (primer 3); 5′-AGGAAGTGTCTCTAGTATCC-3′ (primer 4). The PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN) following the manufacturer's protocol. The nucleotide positions are assigned according to Lindberg *et al*. [19].

2.11. Electrophoretic mobility shift assay

The PCR-derived oligonucleotides, probes 1 and 2, were 5′-end labeled with [γ-^{32}P]ATP (3000 Ci/mmol) using a 5′-end labeling kit (Promega). Binding reactions (10 μL) contained 4% glycerol, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris–HCl (pH 7.5), 0.05 mg/mL poly(dI-dC) (Amersham Biosciences) and 4 μg of nuclear extract (NE) from control or PB-treated mice. After 10 min at room temperature, 1×10^5 cpm of labeled DNA was added and the mixtures were incubated for an additional 20 min at room temperature. In the supershift reactions, 1 μL of antibody was added to the reaction mixture and incubated on ice for 60 min. The monoclonal antibodies used were either the anti-hnRNP A1 (9H10) or the anti-hnRNP 1/PTB (Zymed Laboratories). For the electrophoretic separation of the DNA–protein complexes, the samples were loaded onto a pre-electrophoresed, non-denaturing 4% polyacrylamide (60:1 acrylamide:bisacrylamide) gel in 0.5× TBE (44 mM Tris–HCl, pH 8/44 mM boric acid/1 mM EDTA). The samples were electrophoresed at 10 mA for 270 min, after which the gel was dried and autoradiographed.

2.12. Sequence analysis

The sequence alignments were performed with the GeneWorks 2.5 software (IntelliGenetics, Inc./Oxford Molecular Group).

2.13. Data analysis

All experiments in this study were performed three times. The samples in the Northern analyses, UV crosslinkings and Western blots were performed in duplicate throughout

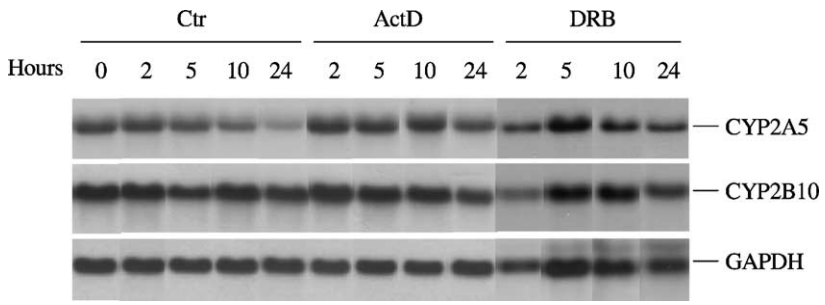


Fig. 1. Effect of ActD and DRB on CYP2A5 mRNA levels in primary hepatocytes. Northern blot analysis was performed using 10 μg of total RNA isolated from primary hepatocytes treated with ActD (4 μM) or DRB (100 μM) during the indicated times. Control cells received DMSO. CYP2A5 and CYP2B10 mRNA levels are shown. GAPDH mRNA levels are shown as controls for RNA loading.

this study. In several cases, different lanes of one gel have been combined into one figure. All samples in the transfections were performed in triplicate, and data presented as means ± standard deviations of the means. Statistical analysis was performed with Student's *t*-test (unpaired).

3. Results

3.1. Effects of transcription inhibition on Cyp2a5 post-transcriptional regulation

In a first set of experiments, transcription was inhibited by treating hepatocytes with 4 μM ActD or 100 μM DRB, two concentrations known to completely block RNA polymerase II activity [20,21]. Fig. 1 shows that CYP2A5 mRNA levels are dramatically reduced in untreated cells during 24 hr of culture whereas they remain unchanged in the presence of ActD or DRB. This indicates that prevention of transcription leads to stabilization of the CYP2A5 mRNA. In contrast, CYP2B10 mRNA levels were not changed in untreated, ActD- or DRB-treated cells, suggesting that this mRNA species is more stable than the CYP2A5 mRNA.

Our previous observations have shown that when the CYP2A5 mRNA is stabilized *in vivo*, its poly(A) tail is elongated, and a strong binding of hnRNP A1 to the CYP2A5 mRNA takes place in the cytoplasm [13]. We observe that ActD has no strong overall effect on the CYP2A5 mRNA poly(A) tail. However, a high molecular weight RNA species, recognized by the CYP2A5 cDNA probe, seems to be induced in the ActD-treated cells (Fig. 2, band marked (∗)). The size of this mRNA fragment is reduced in the presence of oligo dT (band marked (∗∗)) suggesting that it is polyadenylated.

We then investigated the effects of ActD and DRB on the CYP2A5 mRNA binding activity of hnRNP A1 and on its subcellular distribution. After 5 hr of ActD or 24 hr of DRB treatment, we observed a translocation of the binding activity from the nucleus to the cytoplasm (Fig. 3A and B), with a parallel modification of the subcellular localization of hnRNP A1 (Fig. 3C and D).

In conclusion, impaired transcription leads to nucleocytoplasmic translocation of hnRNP A1, to increased CYP2A5 mRNA binding activity of hnRNP A1 in the cytoplasm, and to increased stability of the CYP2A5 mRNA.

3.2. Effect of stimulated transcription of the Cyp2a5 gene on the interaction of hnRNP A1 with CYP2A5 mRNA

In the next series of experiments we wanted to investigate if stimulated transcription of the *Cyp2a5* gene would also affect the subsequent post-transcriptional processes and the subcellular localization of hnRNP A1. For that purpose hepatocytes were treated with PB and cAMP, known transcriptional activators of the *Cyp2a5* [9,22]. As controls, the levels of the PB inducible CYP2B10 and the GAPDH mRNAs were measured. As expected, PB and cAMP increased the CYP2A5 mRNA levels and ActD prevented the induction (Fig. 4A and B), confirming the transcriptional effect of the inducers. The poly(A) tail size of the CYP2A5 mRNA was not affected by PB or by cAMP treatments (data not shown).

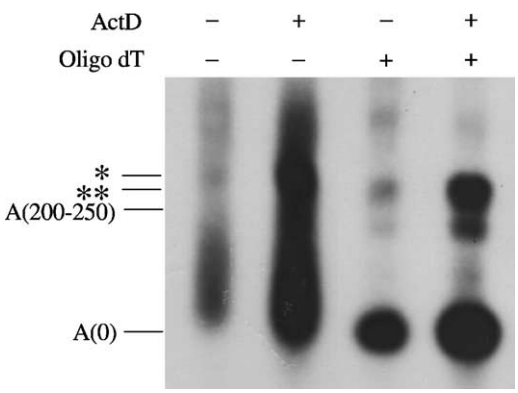


Fig. 2. Effect of ActD on CYP2A5 poly(A) tail length. Cells were treated with 4 μM ActD during 24 hr and the extent of polyadenylation of the CYP2A5 mRNA was investigated in the presence (+) or absence (−) of oligo dT, using an RNase H-based method (for details see Section 2). The approximate number of adenylate residues is indicated. A higher molecular weight RNA, recognized by the CYP2A5 probe, in its adenylated (∗) and deadenylated (∗∗) form is shown.

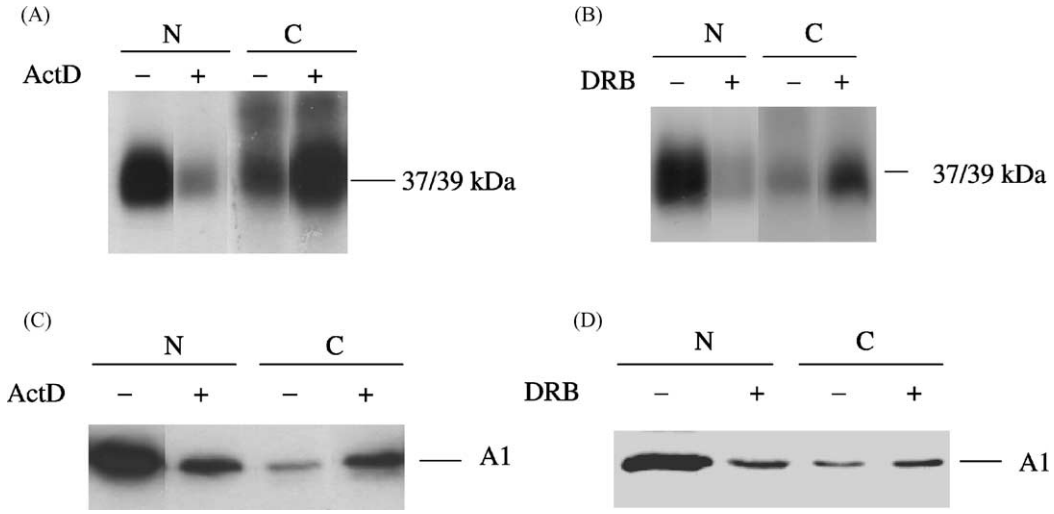


Fig. 3. Effect of ActD and DRB on the RNA-binding activity and subcellular localization of hnRNP A1. 5 μg of nuclear (N) and 10 μg of cytoplasmic (C) proteins prepared from primary mouse hepatocytes were incubated with the radioactive CYP2A5 RNA probe containing the hnRNP A1 binding site, and UV crosslinked. The 37/39 kDa complex is indicated. (A) Cells treated with ActD (4 μM, 5 hr), or with vehicle only (DMSO). (B) Cells treated with DRB (100 μM, 24 hr), or with vehicle only (DMSO). (C) Subcellular localization of hnRNP A1: Western blot analysis was performed using 50 μg of the same proteins as in (A). (D) Western blot analysis was performed using 50 μg of the same proteins as in (B).

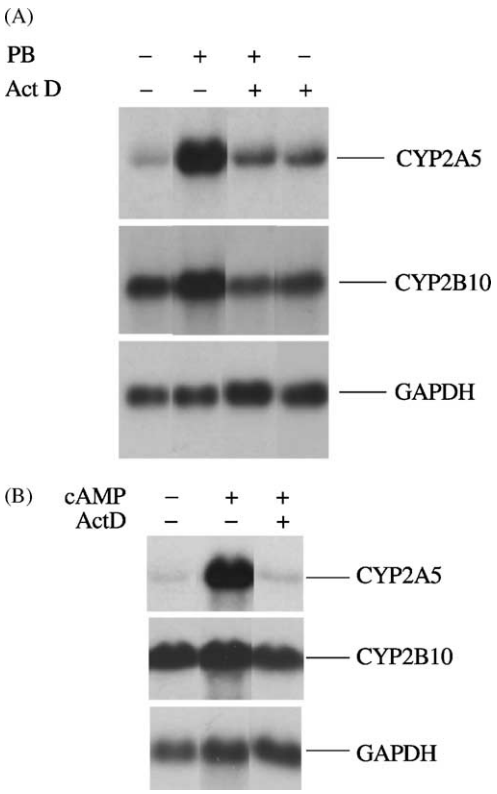


Fig. 4. Effect of transcriptional activators on CYP2A5 and CYP2B10 mRNA levels. (A) Phenobarbital (PB): Northern blot analysis was performed with 20 μg of total RNA from primary mouse hepatocytes, untreated (−) or treated (+) with 1.5 mM PB and 4 μM ActD alone or combined, for 24 hr. Control cells received DMSO. CYP2A5 and CYP2B10 mRNA levels are presented. GAPDH mRNA levels are shown as controls for RNA loading. (B) cAMP: Northern blot analysis was performed using 20 μg of total RNA isolated from primary mouse hepatocytes untreated (−) or treated (+) for 24 hr with 50 μM cAMP or 4 μM ActD alone or combined. CYP2A5 and CYP2B10 mRNA levels are shown. GAPDH mRNA levels are presented as controls for RNA loading.

UV crosslinking studies showed a high nuclear and a low cytoplasmic CYP2A5 mRNA binding activity of hnRNP A1 after the PB and cAMP treatments (Fig. 5A and B). Western blot analysis confirmed that hnRNP A1 is mainly nuclear in untreated, PB- and cAMP-treated cells (data not shown). In conclusion, increasing the transcriptional activity of the *Cyp2a5* gene does not change the subcellular localization of hnRNP A1, which remains essentially nuclear.

3.3. Effect of hnRNP A1 overexpression on the expression of a reporter gene driven by the Cyp2a5 promoter

An expression vector containing the hnRNP A1 coding sequence was used to overexpress hnRNP A1 in mouse primary hepatocytes. In control cells, the same plasmid lacking the hnRNP A1 sequence was used (see Section 2). These plasmids were cotransfected with either a luciferase expression vector bearing the 3 kbp upstream of the *Cyp2a5* transcription start site (*Cyp2a5* promoter-driven pGL3 Basic plasmid) or an SV40 promoter-driven luciferase vector (pGL3 control) for comparison. We observe that hnRNP A1 overexpression leads to a significantly ($P < 0.001$) stronger increase (2.5-fold) in the expression of the *Cyp2a5* promoter-driven luciferase reporter, compared to the SV40 promoter-driven vector (Fig. 6). This result suggests that hnRNP A1 in addition to its post-transcriptional effects on the CYP2A5 mRNA, also plays a role in the transcriptional control of the *Cyp2a5* gene.

3.4. Analysis of hnRNP A1/Cyp2a5 promoter interaction by EMSA

Analysis of the *Cyp2a5* promoter reveals a region of high sequence similarity with the 71 nt hnRNP A1 binding

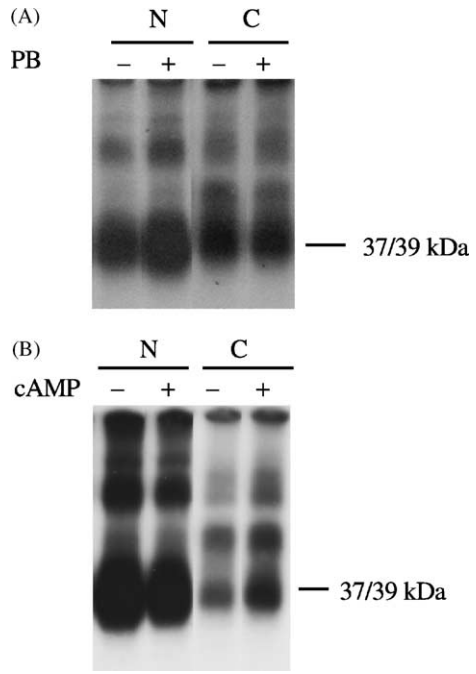


Fig. 5. Effect of transcriptional activators on the RNA-binding activity. (A) Phenobarbital effect on CYP2A5 mRNA binding activity: 5 μg of nuclear (N) and 10 μg of cytoplasmic (C) proteins, prepared from primary mouse hepatocytes, untreated (−) or treated (+) for 24 hr with PB (1.5 mM), were assayed in a UV crosslinking experiment. The 37/39 kDa RNA–protein complex is indicated. (B) cAMP effect on CYP2A5 mRNA binding activity: 5 μg of nuclear (N) and 10 μg of cytoplasmic (C) proteins, prepared from primary mouse hepatocytes untreated (−) or treated (+) with 50 μM cAMP for 24 hr, were assayed by UV crosslinking analysis. The 37/39 kDa complex is indicated.

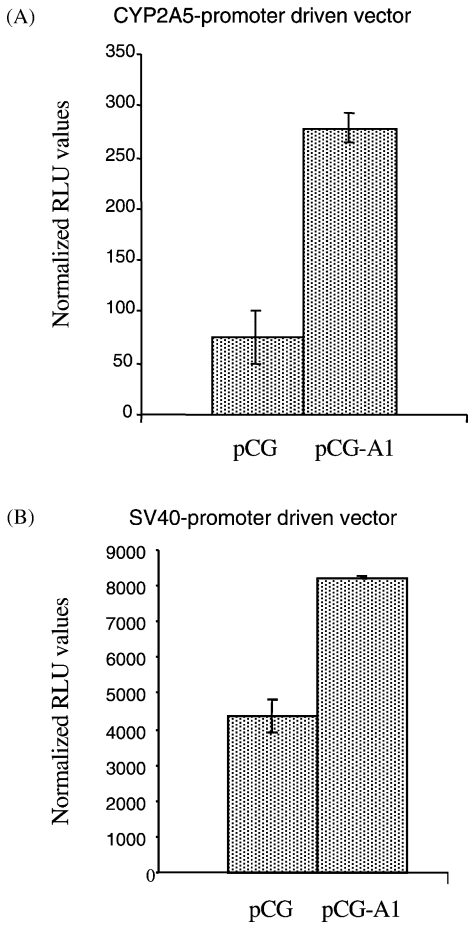
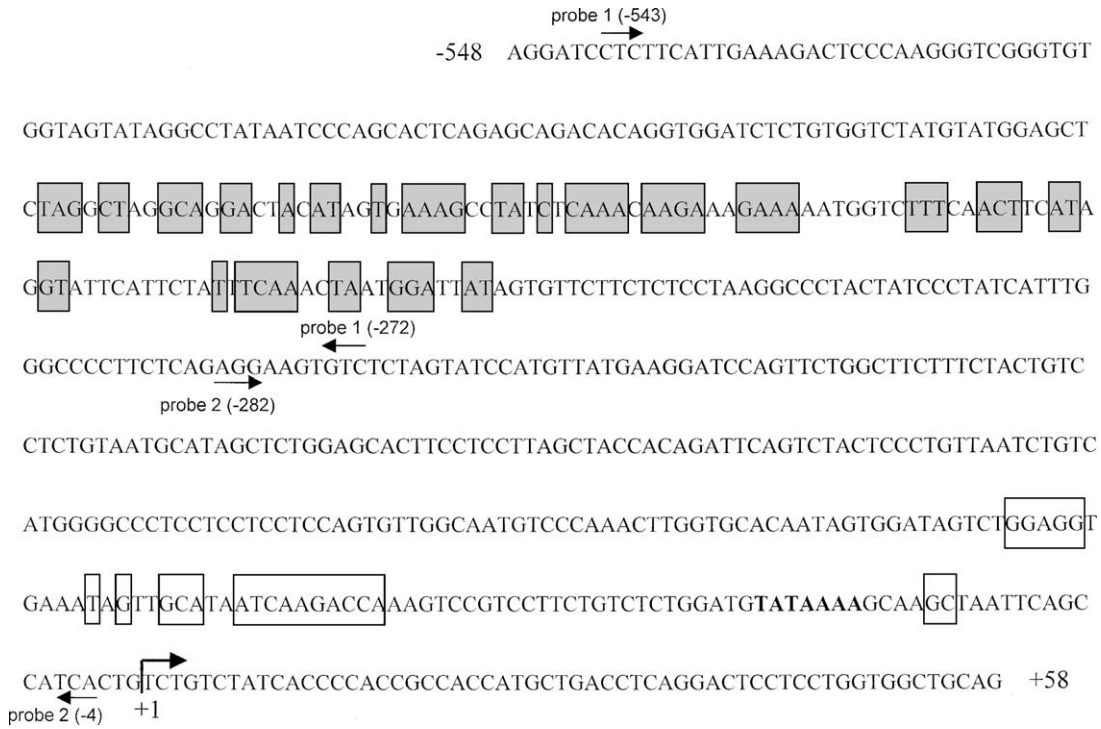


Fig. 6. Effect of hnRNP A1 overexpression on the expression of a reporter gene driven by the *Cyp2a5* promoter. An expression plasmid containing the hnRNP A1 coding region (pCG-A1) was transfected into mouse primary hepatocytes in order to overexpress the hnRNP A1 protein. Control cells received the corresponding plasmid lacking the hnRNP A1 coding region (pCG). (A) A luciferase reporter construct bearing 3 kbp of the *Cyp2a5* promoter region was cotransfected with the pCG and pCG-A1 plasmids. The luciferase expression measured 48 hr after transfection is indicated as normalized relative light units. (B) The SV40 promoter-driven luciferase vector (pGL3 control) was cotransfected with the pCG and pCG-A1 plasmids. The luciferase expression measured 48 hr after transfection is indicated as normalized relative light units.

site in the 3′UTR of CYP2A5 mRNA [13,23] (Scheme 1, shaded boxes). In addition, a 35 bp region in the MHC class II genes to which hnRNP A1 binds *in vivo* during transcriptional upregulation [24], has a sequence similarity to another region in the *Cyp2a5* proximate promoter (Scheme 1, open boxes). This prompted us to investigate the possible interaction of hnRNP A1 with the *Cyp2a5* promoter. We performed electrophoretic mobility shift assays (EMSA) with nuclear extracts from untreated and PB-treated mice using these two regions as probes. Probe 1 spans the region from −543 to −272 in the *Cyp2a5* promoter, and probe 2 the region from −282 to −4 (Scheme 1). As illustrated in Fig. 7 (lanes 2, 3, 7 and 8) complexes were formed with both probes (A and B, indicated by arrows). Proteinase K treatment abolished complex formation, confirming the proteinic character of the complexes (data not shown). The presence of hnRNP A1 in the complexes was investigated in a supershift assay with the monoclonal anti-hnRNP A1 antibody (9H10). A control reaction was performed with a monoclonal anti-hnRNP I/PTB antibody. The hnRNP A1 antibody (Fig. 7) shifted complex A formed with both probe 1 (lane 4) and probe 2 (lane 9), revealing the presence of hnRNP A1 in the DNA–protein complexes. In contrast, the hnRNP I/PTB antibody did not confer any shift (Fig. 7, lanes 5 and 10). Interestingly, PB affected the protein interaction with probe 2. Complex A formation was markedly increased, and complex B diminished.

4. Discussion

Coordination of the various molecular events of gene expression is a central yet poorly understood aspect of gene regulation. In this study, we give evidence for the existence of such a mechanism for a drug inducible gene, *Cyp2a5*, in cultured mouse primary hepatocytes. Inhibition of transcription leads to increased stability of the CYP2A5 mRNA, suggesting that nuclear and cytoplasmic steps are linked. The interaction seems to be mediated by hnRNP A1, which, during impaired transcription, translocates from the nucleus



Scheme 1. Potential hnRNP A1 binding sites in the *Cyp2a5* proximate promoter. A schematic presentation of the −548/+58 bp region in the *Cyp2a5* promoter, with the TATA-box (**TATAAAA**) and transcriptional start site (↱) indicated. The boxed sequences represent the regions of sequence similarity with the 71 nt region (shaded boxes) of the CYP2A5 3′UTR (probe 1) and the 35 bp sequence described by Donev *et al.* [24] (probe 2, open boxes). Sequence alignments were performed with the GeneWorks 2.5 Software.

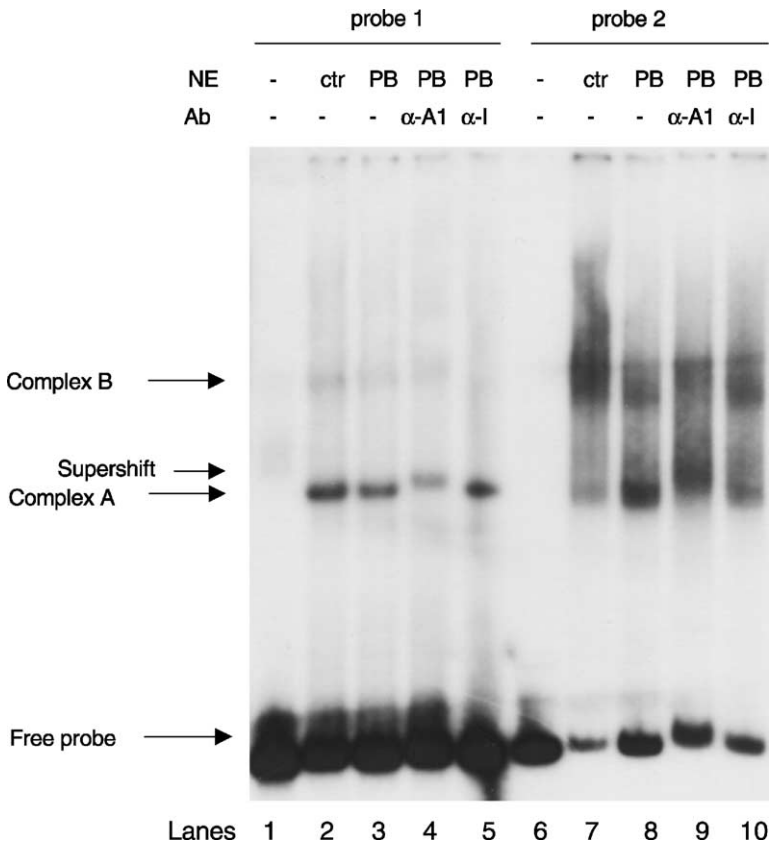


Fig. 7. EMSA analysis of hnRNP A1/*Cyp2a5* promoter interactions. Nuclear extracts (NE) from untreated (ctr) or PB-treated (PB) mice were incubated with radiolabeled probe 1 or 2 (see Scheme 1) in an EMSA assay. When indicated the reactions were incubated with monoclonal anti-hnRNP A1 or anti-hnRNP I/PTB antibodies. The supershift and complexes formed are marked by arrows.

to the cytoplasm. The subsequent stabilization of the CYP2A5 mRNA supports the previously proposed role of hnRNP A1 as a stabilizer of the CYP2A5 transcript [13–15]. Moreover, our present evidence strongly indicates the involvement of hnRNP A1 in the transcription of the *Cyp2a5* gene. The supershift analysis revealed interaction of hnRNP A1 with the *Cyp2a5* promoter which is stimulated upon increased transcription, and overexpression of hnRNP A1 upregulated a *Cyp2a5* promoter-driven recombinant gene. This protein therefore most likely plays a dual role in *Cyp2a5* expression: one in transcription and one in mRNA stabilization. Hence, it may link these two events by shuttling between the nucleus and the cytoplasm.

Two classical transcription inhibitors, ActD and DRB, were used in the studies, and both of these drugs increased the stability of CYP2A5 mRNA. An mRNA stabilizing effect of ActD has been described for a small number of post-transcriptionally regulated genes, such as those coding for the granulocyte macrophage-colony stimulating factor (GM-CSF) or the transferrin receptor [25,26]. Using NIH 3T3 cells, Chen *et al*. [27] showed that ActD and DRB dramatically stabilize transgenic β-globin mRNAs containing the AU-rich element found in the 3′UTR of GM-CSF or c-fos mRNAs. The authors propose that the transcriptional inhibitors could temporarily increase the cytoplasmic level of certain mRNA-binding proteins, possibly certain hnRNPs, which in turn could disrupt the RNA–protein complex necessary for mRNA decay. The work presented here agrees well with this model. Indeed, upon ActD and DRB treatment, the cytoplasmic CYP2A5 mRNA-binding activity increases as a consequence of hnRNP A1 translocation from the nucleus to the cytoplasm. An ActD- or DRB-dependent cytoplasmic accumulation of hnRNP A1 has been observed in other cell systems and has been shown to result from the inhibition of the re-import of hnRNP A1 to the nucleus [28].

As a result of the ActD treatment, we detected a longer and apparently polyadenylated RNA species. To our knowledge, no alternative splicing or alternative polyadenylation of the CYP2A5 mRNA has been reported, and the biological significance of this RNA, in particular, its relation to the enhanced stabilization of CYP2A5 mRNA remains to be investigated.

DRB and ActD inhibit transcription by different mechanisms: ActD prevents the initiation of transcription [29], while DRB causes premature transcriptional termination [30]. Therefore, the observation that both agents produce similar post-transcriptional effects on *Cyp2a5* expression indicates that these effects are independent from the assembly of a functional transcription machinery. Instead, they could be coupled to the appearance of new transcripts in the nucleus. In accordance with this, it has been proposed that depletion of pre-mRNAs in the nucleus causes hnRNP A1 to accumulate in the cytoplasm by disturbing its normal shuttling mechanism [31]. It is worth emphasizing that hnRNP A1 also behaves in this way when the cells are exposed to proinflammatory cytokines [32] or stress stimuli, such as osmotic shock or UV irradiation [33], indicating that translocation of hnRNP A1 could be a way in which cells respond to various types of stress in order to maintain important functions in these conditions. When hepatocytes were exposed to two transcriptional activators of the *Cyp2a5* gene, PB and cAMP, hnRNP A1 remained essentially nuclear. In addition, overexpression of hnRNP A1 upregulated a transiently transfected recombinant gene controlled by the *Cyp2a5* promoter. The supershift experiments established the presence of hnRNP A1 in the protein complex binding to two different *Cyp2a5* promoter regions (probes 1 and 2) *in vitro*. Furthermore, our results imply that the *Cyp2a5* transcriptional inducer, PB, stimulates the interaction of hnRNP A1 with one of the regions. Finally, competition experiments revealed that a known high affinity hnRNP A1-binding sequence, the ‘‘winner’’ RNA [34], was able to specifically compete in the complex formation, confirming the presence of hnRNP A1 in the protein–DNA complex (unpublished observations). The results strongly suggest that hnRNP A1 is involved in the transcription of the *Cyp2a5* gene, and may participate in the stimulated transcription by PB. A role of hnRNP A1 in the transcriptional activation has been reported for other genes, such as the thymidine kinase and MHC class II genes [12,24].

In conclusion, we have in this study revealed an interdependence between transcriptional activity and the stability of CYP2A5 mRNA. The apparent crosstalk observed agrees with a model proposed by Wilkinson and Shyu [2] where the integration of transcription with the subsequent steps is mediated by multifunctional regulators having functions both in the nucleus and the cytoplasm. It appears that hnRNP A1 is an attractive candidate in coordinating the nuclear and cytoplasmic steps of *Cyp2a5* expression [14,23,28]. In support of this, Visa *et al*. [35] have shown that the homologue of the hnRNP A1, hrp36, is associated with mRNA from transcription to translation.

Why is the *Cyp2a5* gene regulated so that its expression is maintained in conditions where the transcription capacity of the cell is impaired? This particular type of regulation has been described for other genes, such as cyclin D3 [36], cyclooxygenase-2 [37] and GM-CSF [25]. Based on these reports it can be speculated that one purpose is to maintain important cell functions during disturbed transcription, in order for the cells to survive. Whether the detoxification capacities of CYP2A5 qualify for such important functions or if the enzyme has additional functions, vital for the cell, is not yet known.

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