

Interplay between hnRNP A1 and a *cis*-acting element in the 3' UTR of CYP2A5 mRNA is central for high expression of the gene

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Abstract Our previous evidence suggests that heterogeneous nuclear ribonucleoprotein (hnRNP) A1 plays a part in the regulation of the *Cyp2a5* gene by interacting with the 3' untranslated region (UTR) of the CYP2A5 mRNA. However, the exact role of this interaction is not clear. The aim of the present work was to gain further insight into the regulation process of *Cyp2a5*. For this purpose the 3' UTR of CYP2A5 was fused to the coding region of luciferase mRNA. Luciferase recombinants containing either the full length 3' UTR, or the 3' UTR lacking a previously described 71 nucleotide (nt) region (the hnRNP A1 primary binding site), were transiently expressed in cells expressing or lacking hnRNP A1. The expression of the luciferase recombinants was examined both at mRNA and enzyme activity levels. The results disclosed that the presence of hnRNP A1 was required for the high expression of the recombinant carrying the full length 3' UTR of CYP2A5. Deletion of the hnRNP A1 primary binding site dramatically modified the expression pattern: the mRNA levels and luciferase activities of the deletion mutant were independent from hnRNP A1. These results conclusively demonstrate that the 71 nt region in the 3' UTR of CYP2A5 mRNA can confer hnRNP A1-dependent regulation to a gene. In addition, comparison of RNA levels and luciferase activities suggested that regions flanking the hnRNP A1 binding site could regulate translation of the CYP2A5 mRNA. These results are consistent with a model in which the binding of hnRNP A1 to the 71 nt putative hairpin-loop region in the CYP2A5 mRNA 3' UTR upregulates mRNA levels possibly by protecting the mRNA from degradation.

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Key words: CB3 cells; Destabilizing element; Gene regulation; Heterologous reporter gene; Post-transcriptional control

1. Introduction

The cytochrome *P450* mono-oxygenases (CYPs) constitute a large and diverse family of enzymes responsible for the initial oxidation of various lipophilic compounds, including xenobiotics and endogenous substrates. These enzymes are

often inducible, and their expression can be regulated at different levels, such as gene transcription, pre-mRNA processing, mRNA and protein stabilization [1]. The discovery of the 'orphan' nuclear receptor superfamily notably advanced the understanding of the mechanisms by which xenochemicals transcriptionally affect the expression of CYP genes [2,3]. The post-transcriptional regulation of CYP genes, in particular the possible *cis*-acting elements and *trans*-acting proteins involved, however, remain to be elucidated. The interaction of proteins with regulatory elements in the 3' untranslated region (3' UTR) of mRNA has been described in a wide variety of systems [4]. These studies suggest multiple roles of the 3' UTR of eukaryotic mRNA in determining mRNA localization [5], controlling polyadenylation [6], regulating mRNA stability [7,8] and controlling translation initiation [9].

We have studied the molecular mechanisms involved in the post-transcriptional regulation of the *Cyp2a5* gene. Previous work has revealed that *Cyp2a5* is upregulated through mRNA stabilization [10]. In an effort to characterize the regulatory factors involved, we have found that the binding of a 37/39 kDa protein to a 71 nucleotide (nt) long putative hairpin-loop in the CYP2A5 3' UTR is stimulated upon stabilization of the mRNA [11,12]. Geneste et al. [11] also established that during stabilization an elongation of the CYP2A5 mRNA poly(A) tail takes place, suggesting that the binding of the 37/39 kDa protein stabilizes the CYP2A5 mRNA by controlling the length of its poly(A) tail. The 37/39 kDa protein has recently been identified as the heterogeneous nuclear ribonucleoprotein (hnRNP) A1, and overexpression studies conducted in primary mouse hepatocytes imply that hnRNP A1 is an important regulator of the *Cyp2a5* gene expression in vivo [13]. However, the exact role of hnRNP A1 in the *Cyp2a5* regulation remains to be elucidated. The aim of this work was to investigate how hnRNP A1 regulates the *Cyp2a5* expression, in particular what role the interaction of hnRNP A1 with the 71 nt long putative hairpin-loop at the 3' UTR of CYP2A5 mRNA plays in the regulation process. For this purpose we used the CB3+/- cells, expressing or not the hnRNP A1 [14]. The full length CYP2A5 3' UTR, or the 3' UTR lacking the 71 nt hairpin-loop region was fused to the coding region of luciferase mRNA, and these recombinants were transiently expressed in CB3+ and CB3- cells. The results show that interaction of hnRNP A1 with the 71 nt hairpin-loop is critical for a high expression of the gene. In the absence of hnRNP A1 this hairpin-loop region seems to promote degradation of the mRNA. Moreover, deletion of the binding site leads to higher mRNA levels that can no longer be regulated by hnRNP A1. These results are consistent with

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Abbreviations: CYP, cytochrome *P450*; UTR, untranslated region; hnRNP, heterogeneous nuclear ribonucleoprotein; delta71, CYP2A5 3' UTR lacking the 71 nt hnRNP A1 primary binding site; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase

a model proposed for other genes where hnRNP A1 upregulates mRNA levels by preventing a nuclease attack at a destabilizing element.

2. Materials and methods

2.1. Cell cultures

CB3+ and CB3− cells were maintained in MEM medium alpha supplemented with 10% (v/v) fetal bovine serum and 800 µg/ml genetecin (Gibco®; Invitrogen AB, Sweden). The cells were grown in suspension, as described by Ben-David et al. [14].

2.2. Luciferase reporter gene constructs

The luciferase reporter vector, pGL3 control, was purchased from Promega (Scandinavian Diagnostic Services, Falkenberg, Sweden). Downstream of the luciferase gene, at the *Xba*I site, either the full length CYP2A5 3′ UTR or the delta71 CYP2A5 3′ UTR (lacking the 71 nt hnRNP A1 primary binding site) was introduced (Scheme 1). Polymerase chain reaction (PCR) was used to generate the CYP2A5 3′ UTR DNA fragments. To allow subcloning of the fragments into the pGL3 control vector, the *Xba*I restriction site (TCTAGA) was introduced in the forward primer, and the *Avr*II restriction site (CCTAGG) in the reverse primer. Oligonucleotides are shown in sense orientation with the restriction sites underlined. Nucleotide positions referring to the CYP2A5 sequence were assigned according to Squires and Negishi [15].

The forward primer, common to both constructs, was: CYP2A5 1486–1689, ACACACTCTAGAGCCTGGGCTGCATGAGGTTAAAGGGAATG. The reverse primers were the following: Full length CYP2A5 1689–1486, ACACACCCTAGGATTCCTATTGACAACATAGT; Delta71 CYP2A5 1689–1655//1584–1486, ACACACCCTAGGTTGCCATAAATAATATCTACTTTTATTATTATT/TCAGTGTCCTCTGTTTCTTCTGTACCTTTGACC.

The PCR-amplified fragments were double digested with *Xba*I and *Avr*II before ligation into the *Xba*I digested pGL3 control vector. All constructs were sequenced to ascertain the right orientation in the vector, using Dye-labelled Terminator for the sequence reaction and an ABI377 DNA sequencer for analysis.

2.3. Transfection experiments

The luciferase reporter gene constructs were transiently transfected into CB3+ and CB3− cells with the DMRIE-C Reagent transfection system (Gibco®, Invitrogen AB, Sweden) and according to the manufacturer's protocol. Co-transfection with a β -galactosidase plasmid, pCMV-SPORT- β -gal (Gibco®, Invitrogen AB, Sweden) allowed monitoring of transfection efficiencies.

2.4. Luciferase assay

Transfected CB3+/- cells were assayed for luciferase activity 48 h after transfection. All reagents required for the luciferase assay were from Promega, SDS, Sweden. The cells were lysed and harvested in reporter lysis buffer (RLB) and immediately put on dry ice. Before luminescence reading, the thawed cell extracts were centrifuged at 9500 \times g for 2 min; 30 µl cell extract was added to 100 µl luciferase reagent, and the luminescence in relative light units (RLU) was read in a TD-20/20 luminometer (Turner Designs, Scandinavian Diagnostic Services, Sweden). The RLU values were normalized to the β -galactosidase activities.

2.5. β -Galactosidase assay

To 50 µl cell extracts, 250 µl buffer Z (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1.0 mM MgSO₄, 50 mM β -mercaptoethanol) was added. The samples were incubated for 2 h at room temperature after addition of 50 µl of a 4 mg/ml ONPG (*O*-nitrophenyl- β -D-galactopyranoside, Sigma-Aldrich Sweden AB). Subsequently, the reaction was stopped by adding 250 µl of 1.0 M Na₂CO₃ and the absorbance measured spectrophotometrically at 420 nm.

2.6. Nuclear and cytoplasmic proteins

Cells (5 \times 10⁶) were centrifuged at 2000 \times g for 3 min and washed twice in phosphate buffered saline (PBS). Following this, the pellet was resuspended in 400 µl buffer A (10 mM HEPES-KOH pH 7.9; 10 mM KCl; 1.5 mM MgCl₂; 0.5 mM dithiothreitol (DTT); 0.2 mM phenylmethylsulfonyl fluoride (PMSF); 10 µg/ml leupeptin and 0.4%

Igepal) and left on ice for 60 min. After 30 s of vortexing, at maximum speed, cells were homogenized by pestling (10 strokes \times 3) and centrifuged at 11 000 \times g and 4°C for 10 min. The supernatant containing the cytoplasmic proteins was stored at −80°C. The remaining pellet containing the nuclei was resuspended in 100 µl cold buffer B (20 mM HEPES-KOH pH 7.9; 25% glycerol; 1.5 mM MgCl₂; 420 mM NaCl; 0.2 mM ethylenediamine tetraacetic acid (EDTA); 0.5 mM DTT; 0.5 mM PMSF; and 0.4% Igepal) and stirred with a magnetic stirrer for 30 min at 4°C. The suspension was homogenized by pestling (10 strokes \times 2) and centrifuged at 11 000 \times g for 15 min at 4°C. The nuclear proteins contained in the supernatant were stored at −80°C.

2.7. Western blot

Proteins (30 µg) were isolated as described, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12%) and blotted onto a nitrocellulose membrane, Hybond®-C extra (Amersham Biosciences, Uppsala, Sweden). A monoclonal anti-hnRNP A1 antibody, 9H10, kindly provided by Dr. G. Dreyfuss (Howard Hughes Medical Institute, University of Philadelphia, USA) was used in a 1:1000 dilution. All solutions contained 5% non-fat dry milk and the detection was performed with the ECL Western blotting analysis system (Amersham Biosciences, Uppsala, Sweden).

2.8. Northern blot

Total RNA from transfected CB3+/- cells was isolated using the Qiagen Miniprep kit (Qiagen, VWR International, Stockholm, Sweden). RNA was run on a 1.2% agarose/formaldehyde gel and transferred onto a Hybond®-N nylon membrane (Amersham Biosciences, Uppsala, Sweden). The amount of RNA loaded was determined according to the transfection efficiency, as described in more detail in Section 3. Following transfer, the membrane was UV crosslinked and hybridized overnight at 65°C in a modified Church buffer (0.25 M phosphate buffer, 7% SDS, 1 mM EDTA) with [α -³²P]dCTP-labelled cDNA (1.7 \times 10⁷ cpm) corresponding to the luciferase-coding region. Radiolabelling was performed with the Megaprime labelling kit (Amersham Biosciences, Uppsala, Sweden).

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

2.9. Expression and purification of recombinant histidine-tagged hnRNP A1

The histidine-tagged recombinant hnRNP A1, generously provided by Dr. J.-P. Kreivi (Uppsala Biomedical Centre, Uppsala University, Sweden), was expressed in *Escherichia coli* BL21(DE3)/pLysS as described by Mayeda et al. [16] and purified with a HiTrap Chelating HP affinity column (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.10. Synthesis of radiolabelled RNA

The 71 nt RNA probe (containing the hnRNP A1 primary binding site) was synthesized by in vitro transcription of CYP2A5 cDNA amplified by PCR [12]. The PCR-amplified cDNA was transcribed with T7 RNA polymerase in the presence of [α -³²P]UTP using standard protocols (Promega Corporation, 'Protocols and Applications Guide', 3rd Edn., 1996).

2.11. UV crosslinking

A detailed protocol of the reaction is described by Geneste et al. [11]. In brief, 5 µg of nuclear and 10 µg of cytoplasmic proteins were used in the binding reactions. The samples were exposed to UV light for 20 min in a Spectrolinker XL-1000 UV crosslinker (Spectronics, Westbury, NY, USA). Unprotected RNA was digested with 2 µg of RNase A at 37°C for 20 min. The samples were denatured under non-reducing conditions and the proteins were separated by SDS-PAGE (12%). Ultimately, the gel was dried and autoradiographed overnight.

2.12. Immunoprecipitation

Immunoprecipitation was performed according to Hamilton et al. [17]. The UV crosslinked CYP2A5 RNA/protein complexes (14 µg cytoplasmic proteins and 7 µg nuclear proteins) were incubated with a 1:500 dilution of the anti-hnRNP A1 antibody, 9H10, and subsequently immunoprecipitated with protein A-Sepharose beads (Amer-

sham Biosciences, Uppsala, Sweden). After washing, the immunoprecipitated complexes were denatured and separated by SDS-PAGE (12%), and radiolabelled complexes were detected with autoradiography.

3. Results

3.1. Expression of hnRNP A1 and its CYP2A5 mRNA binding activity in CB3+/- cells

The CB3 cells have lost the ability to express the hnRNP A1 protein due to a retroviral insertion in one allele of the *hnnpa1* gene, and a loss of the other allele [14]. These cells have been transformed with a vector containing the mouse hnRNP A1-coding region (CB3+), or with the corresponding empty vector (CB3-) [14]. A first set of experiments was conducted in order to confirm the lack of hnRNP A1 expression and CYP2A5 mRNA binding activity in CB3- cells. Western blot analysis with a monoclonal antibody raised against the hnRNP A1 revealed that the protein is expressed in CB3+, but not in CB3- cells (Fig. 1A), a result in accordance with previous reports on the CB3+/- cells [14]. In addition, a UV crosslinking assay with the 71 nt hairpin-loop region of the CYP2A5 mRNA as a probe, containing the primary binding site of the hnRNP A1 [11], confirmed that a UV crosslinked complex was obtained only with nuclear and cytoplasmic protein extracts from CB3+ cells [13] and this complex was identical in size to that formed by a purified recombinant hnRNP A1 and the 71 nt RNA probe (Fig. 1B). With immunoprecipitation, the complex formed with CB3+ cell extracts was shown to contain the hnRNP A1 (Fig. 1C). In both UV crosslinking and immunoprecipitation experiments larger size protein complexes were detected, most probably due to hnRNP A1 homomerization as described by Cartegni et al. [18] and Kim et al. [19].

3.2. Role of the CYP2A5 3' UTR for the luciferase gene expression in the CB3+ and CB3- cells

Having validated that exclusively the CB3+ cells expressed the hnRNP A1, and that the binding to the CYP2A5 mRNA was maintained in these cells, both CB3+ and CB3- cells were transiently transfected with luciferase reporter constructs (Scheme 1), in order to see how interaction of hnRNP A1 with the 3' UTR of the CYP2A5 mRNA could control gene expression. Total RNA and protein from transfected cells were isolated 48 h post-transfection, a time point at which the transient expression of the reporter constructs was at its peak (data not shown). The expression of the recombinant genes was first analyzed at the mRNA level, by Northern blot (Fig. 2). The luciferase mRNA levels were quantified with the NIH-Image program (see Section 2). The amount of RNA used in the analysis was normalized for variances in the transfection efficiencies using β -galactosidase activity values. As a control for correct normalization we measured the level of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA, which is expected to vary inversely in proportion to the β -galactosidase activities. Densitometric analysis showed that the GAPDH mRNA levels reflected the correction performed in the β -galactosidase normalization (data not shown). The recombinant luciferase mRNA was detected at the expected size of ~3 kb. Luciferase transcripts of higher molecular weight were also present and their expression seemed to follow that of the luciferase mRNA. We did

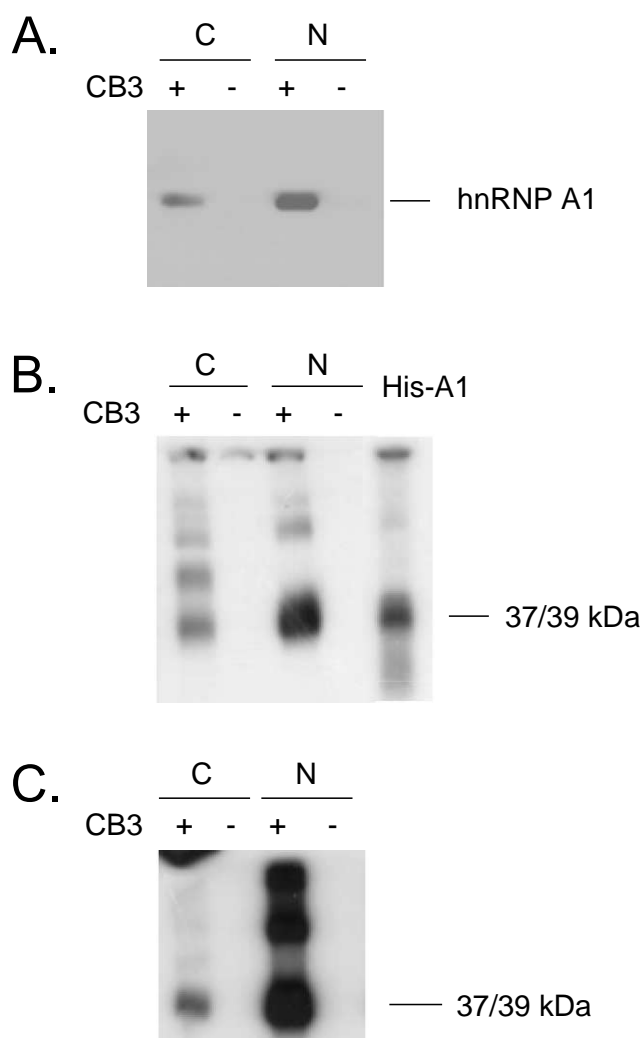
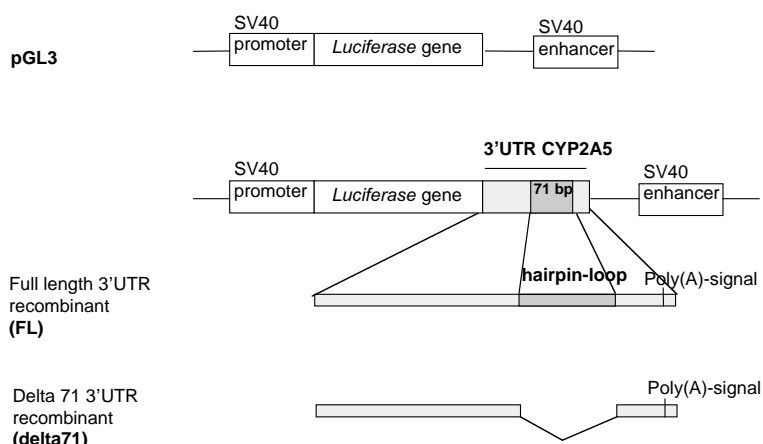


Fig. 1. Characterization of hnRNP A1 in CB3+ and CB3- cells: A: Western blot. Nuclear and cytoplasmic proteins (30 μ g) from CB3+ and CB3- cells were resolved by SDS-PAGE (12%) and transferred onto a nitrocellulose membrane. The hnRNP A1 protein was detected with the monoclonal anti-hnRNP A1 antibody (9H10). B: UV crosslinking of nuclear and cytoplasmic proteins from CB3+ and CB3- cells and the 71 nt CYP2A5 RNA probe. UV crosslinking was performed with 5 μ g of nuclear (N) or 10 μ g of cytoplasmic (C) proteins from CB3+ and CB3- cells, or 10 μ g of the recombinant histidine-tagged hnRNP A1 (his-A1), and the radiolabelled 71 nt CYP2A5 RNA probe. The 37/39 kDa complex is indicated. C: Immunoprecipitation of RNA/protein complexes. UV crosslinking was performed on 7 μ g of nuclear (N) or 14 μ g of cytoplasmic (C) proteins from CB3+ and CB3- cells incubated with the radiolabelled 71 nt CYP2A5 RNA probe, corresponding to the hnRNP A1 primary binding site. The RNA/protein complexes were immunoprecipitated with the anti-hnRNP A1 antibody (9H10), and resolved by SDS-PAGE (12%). The hnRNP A1-containing 37/39 kDa complex is indicated.

not investigate their identity further but these transcripts may be due to cryptic transcription start sites within the plasmid [20].

When the luciferase recombinant containing the full length CYP2A5 3' UTR was transfected into the CB3+ cells, higher luciferase mRNA levels were detected compared to the control luciferase mRNA not containing the CYP2A5 3' UTR (Fig. 2). However, transfection into CB3- cells resulted in markedly reduced levels of the CYP2A5 3' UTR-containing



Scheme 1. Schematic presentation of the CYP2A5 luciferase reporter recombinants. The 71 nt putative hairpin-loop is indicated. The different parts of the CYP2A5 3' UTR were introduced downstream of the luciferase-coding region, at the *Xba*I restriction site.

luciferase mRNA (Fig. 2). In fact, the mRNA level was below the control pGL3 mRNA. Deletion of the hnRNP A1 binding site from the 3' UTR dramatically changed this pattern: in this case no difference was observed between the levels of the control pGL3 mRNA and the delta71 3' UTR-containing luciferase mRNA, in either of the two cell lines (Fig. 2), suggesting that hnRNP A1 no more could affect the recombinant RNA levels.

The luciferase construct containing the full length CYP2A5 3' UTR equally resulted in a strongly elevated luciferase activity when expressed in the CB3+ cells, compared to the control pGL3 vector, but transfection of these constructs into the CB3− cells gave similarly low enzyme activities (Fig. 3). Notably, deletion of the hnRNP A1 binding site from the 3' UTR resulted in a higher enzyme activity/mRNA ratio as compared to that obtained with the pGL3

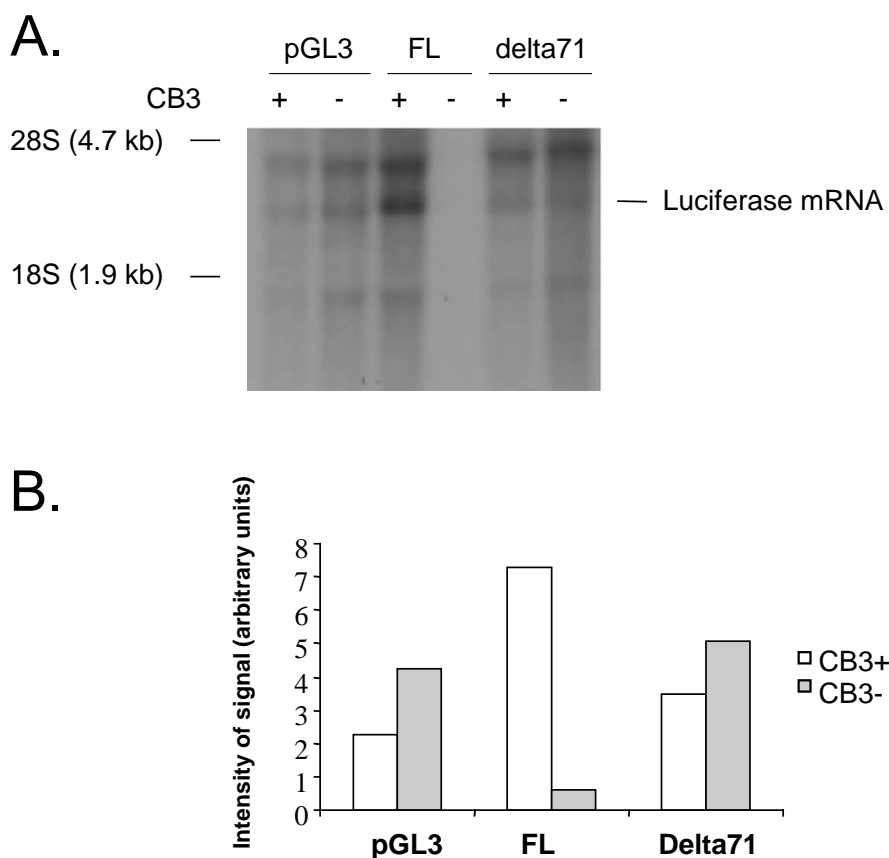


Fig. 2. A: Expression of luciferase recombinants in CB3+ and CB3− cells 48 h after transfection analyzed by Northern blot. Normalized amounts of RNA (as described in Section 3) were hybridized with a radiolabelled probe recognizing the luciferase-coding region. The luciferase mRNA is detected at the expected size of ~3 kb. B: Quantification of pGL3, full length CYP2A5 3' UTR (FL) and delta71 luciferase mRNA amounts in CB3+ and CB3− cells.

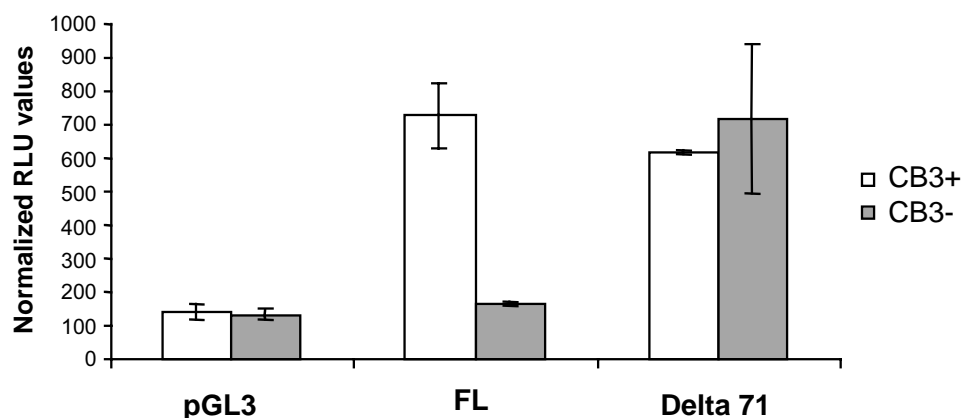


Fig. 3. Luciferase activities in CB3+ and CB3- transfected cells. Protein extracts from cells transfected with the pGL3, full length CYP2A5 3' UTR (FL) and delta71 luciferase reporter constructs were analyzed in a luciferase assay. Cells were harvested 48 h after transfection. Values are means \pm standard deviations; $n=2$. The decreased luciferase activity of the full length CYP2A5 3' UTR (FL) construct in CB3- compared to CB3+ cells was statistically significant ($P < 0.05$).

control. This suggests that regions flanking the hairpin-loop contribute to efficient translation (Fig. 3).

4. Discussion

In this work, the interplay between the hnRNP A1 protein and the putative 71 nt hairpin-loop of the 3' UTR of CYP2A5 mRNA was demonstrated to be essential for the expression of the CYP2A5 3' UTR-containing luciferase gene. In CB3+ cells where the hnRNP A1 was present, the full length CYP2A5 3' UTR-containing reporter gene was highly expressed, both at mRNA and enzyme levels, whereas in CB3- cells lacking hnRNP A1, the same gene was expressed at low levels. These results strongly suggest that the 71 nt region is a *cis*-acting element, which through interaction with the hnRNP A1 controls the levels of the 3' UTR-containing mRNA. Since the 3' UTR-containing mRNA was expressed at levels even lower than the control mRNA in the absence of hnRNP A1, the 71 nt region seemed to act as a destabilizing element protected by the hnRNP A1. The present findings are in accordance with a model postulated by Chen et al. [21], stating that the binding of hnRNP proteins to sequences in the 3' UTR of *c-fos* and granulocyte macrophage-colony-stimulating factor (GM-CSF) mRNAs results in a stabilizing effect on the mRNA. The suggested mechanism is that hnRNPs by binding to regions in the 3' UTR hinder binding of proteins with an mRNA destabilizing function. Our results support this model: the binding of hnRNP A1 to the 71 nt primary binding site in the 3' UTR of CYP2A5 mRNA could protect the mRNA from RNases and thereby maintain the high level of expression of the gene. Recent work on the expression of *Cyp2a5* in primary mouse hepatocytes [13] has revealed that the level of the CYP2A5 transcript increases when hnRNP A1 is overexpressed, another piece of evidence in support of our model in which hnRNP A1 by binding to the 3' UTR of CYP2A5 mRNA has a stabilizing effect on the mRNA. In further support of such a model, hnRNP A1 has newly been reported to protect telomeric sequences against degradation by nucleases [22], again stressing the role of hnRNP A1 as a possible factor protecting certain nucleic acids against nuclease attack.

The delta71 recombinant gave similar luciferase activities in both CB3+ and CB3- cells, as well as comparable mRNA levels. Clearly, the expression of this construct was not dependent on hnRNP A1. Interestingly, despite equally low pGL3 and delta71 mRNA levels in both cell lines, the paralleling enzyme activity was higher for the delta71 recombinant compared to the pGL3. This suggests that the difference in expression is at the translational level; hence the CYP2A5 3' UTR sequences flanking the hairpin-loop in the mRNA may stimulate translation. The role of the 3' UTR in translation has been extensively studied [23,24], and this region of the mRNA is recognized as enhancing the initiation of translation in many mRNA species, by binding factors that interact with the cap structure at the 5' end of the mRNA molecule [25]. No such factors have so far been identified for the CYP2A5.

The results presented here provide conclusive evidence for hnRNP A1 being a central player in the regulation of the *Cyp2a5* expression through its interaction with the hairpin-loop at the 3' UTR. They also suggest that hnRNP A1 works by antagonizing a CYP2A5 mRNA destabilizing factor. The results thus provide a mechanistic explanation for our previous data [11–13]. While this mode of regulation of mRNA stability has been shown for other genes [26] no data on the existence of such factors in the case of *Cyp2a5*, nor any other *Cyp* gene, are available. Experiments aiming at identifying and characterizing factors responsible for CYP2A5 mRNA degradation are ongoing in our research group.

Earlier work has shown that *Cyp2a5* is upregulated in the liver by different conditions causing cellular stress, such as liver injury [27] and viral infection [28]. As the hnRNP A1 also has been shown to be activated in certain stress conditions [29,30], it is tempting to speculate that this protein plays a role in the upregulation of *Cyp2a5* (and perhaps other genes) in various liver pathologies. To investigate this possibility and to study the processes involved is a goal of our future research.

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