DMD Fast Forward. Published on March 10, 2008 as doi:10.1124/dmd.107.019380

TITLE PAGE

ATF5 is a highly abundant liver-enriched transcription factor that cooperates with CAR in the transactivation of *CYP2B6*: Implications in hepatic stress responses.

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DMD Fast Forward. Published on March 10, 2008 as DOI: 10.1124/dmd.107.019380
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Running Title:

ATF5 regulates *CYP2B6*

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Number of text pages: 38

Number of tables: 2

Number of figures: 9

Number of references: 44

Number of words in abstract: 260

Number of words in introduction: 639

Number of words in discussion: 1582

Non-standard abbreviations: **Ad**, adenovirus; ***AldoB***, aldolase B; **ATF**, activating transcription factor; ; **bZIP**, basic region leucine zipper; **C/EBP**, CCAAT/enhancer-binding protein; **CAR**, constitutive androstane receptor; **CHOP**, C/EBP homologous

protein ; **CITCO**, 6-(4-chloropheny-l)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime; **CREB**, cAMP response element binding proteins; **CYP**, cytochrome P450; **ER**, endoplasmic reticulum; **HNF**, hepatic nuclear factor; **KRB**, Krebs-Ringer-Bicarbonate buffer; **LETF**, liver enriched transcription factor; **m.o.i**, multiplicity of infection; **PB**, phenobarbital; **PEPCK**, phosphoenolpyruvate carboxykinase; **Q-RT-PCR**, quantitative real time polymerase chain reaction; **uORF**, upstream open reading frame; **UTR**, untranslated region.

ABSTRACT

Activating transcription factor 5 (ATF5) is a member of the ATF/CREB family, which has been associated with differentiation, proliferation and survival in several tissues and cell types. However, its role in the liver has not yet been investigated. We demonstrate herein that ATF5 is a highly abundant liver-enriched transcription factor whose expression declines in correlation with the level of dedifferentiation in cultured human hepatocytes and cell lines. Re-expression of ATF5 in human HepG2 cells by adenoviral transduction resulted in a marked selective up-regulation of *CYP2B6*. Moreover, adenoviral co-transfection of ATF5 and CAR caused an additive increase in CYP2B6 mRNA. These results were confirmed in cultured human hepatocytes, where the cooperation of ATF5 and CAR not only increased *CYP2B6* basal expression but also enhanced the induced levels after PB or CITCO. Comparative sequence analysis of ATF5 and ATF4, its closest homologue, showed a large conservation of the mRNA 5´-UTR organization, suggesting that ATF5 might be up-regulated by stress-responses through a very similar translational mechanism. To investigate this possibility, we induced endoplasmic reticulum stress by means of amino acid limitation or selective chemicals, and assessed the time-course response of *ATF5* and *CYP2B6*. We found a post-transcriptional up-regulation of ATF5 and a parallel induction of CYP2B6 mRNA. Our findings uncover a new liver-enriched transcription factor coupled to the differentiated hepatic phenotype that cooperates with CAR in the regulation of drug-metabolising *CYP2B6* in the liver. Moreover, ATF5, and its target gene *CYP2B6*, are induced under different stress conditions, suggesting a new potential mechanism to adapt hepatic cytochrome P450 expression to diverse endobiotic/xenobiotic harmful stress.

INTRODUCTION

ATF5, also known as ATFx and ATF-7, is a transcription factor of the ATF/CREB family, whose members contain related basic-region leucine zipper (bZIP) DNA-binding domains. ATF proteins can bind as homo- or heterodimers to a consensus DNA-binding site known as cAMP-responsive element (CRE) (Hai and Hartman, 2001). ATFs diverge considerably outside their bZIP DNA binding regions, and it is the non-conserved portions that contain the diverse transcriptional regulatory domains. More than 20 different mammalian cDNAs with the prefix ATF or CREB have been described, and a large functional diversity has been found (Hai and Hartman, 2001). A comparison of the primary structures of bZIP transcription factors revealed that ATF5 is closely related to ATF4 which is involved in the integration of stress responses, such as the unfolded protein response, or those induced by amino acid deprivation and oxidants (Harding et al., 2003).

ATF5 has been associated with multiple distinct processes such as the differentiation of neural progenitor cells (Angelastro et al., 2003), the repression of cAMP-induced transcription in JEG3 choriocarcinoma cells (Pati et al., 1999), the inhibition or promotion of apoptosis (Persengiev et al., 2002; Wei et al., 2006), the response to amino acid limitation in HeLa cells (Watatani et al., 2006), and the circadian rhythm in adrenal medulla chromaffin cells (Lemos et al., 2007). ATF5 has been extensively investigated in the brain (Angelastro et al., 2005; Mason et al., 2005; Angelastro et al., 2006) but few studies have investigated the functionality of ATF5 in other tissues and cell types (Pati et al., 1999; Watatani et al., 2006; Lemos et al., 2007; Wang et al., 2007). Noteworthy, tissue expression analyses by northern blot suggest that ATF5 is efficiently expressed in the adult liver (Peters et al., 2001; Hansen et al., 2002; Forgacs et al., 2005). Despite this however, the role of ATF5 in human hepatic cells has not yet been investigated.

Cytochromes P450 (CYP) are a superfamily of monooxygenases, playing a key role in the oxidative metabolism of biological signaling molecules such as steroids, and xenochemicals including pharmaceutical drugs and environmental contaminants (Nelson et al., 1996). CYP enzymes generally play a protective role against these xenochemicals, and also in the stress responses elicited by bile acids in cholestasis and by hyperbilirubinemia (Goodwin and Moore, 2004; Qatanani and Moore, 2005). Many CYPs are highly expressed in the liver, and recent studies have shown that their hepatic-specific expression is primarily governed at the transcriptional level by the concerted action of both liver-enriched and ubiquitous transcription factors (Akiyama and Gonzalez, 2003). In addition, the CYP expression can be transcriptionally induced by exposure of cells to xenochemicals as an adaptive response to increase the removal of potentially toxic xenobiotics and endobiotics. In this context, CAR, a nuclear receptor with a high expression level in the liver, mediates the induction of CYPs and other genes involved in the humoral response to both endobiotic and xenobiotic harmful stress (Qatanani and Moore, 2005). A prototypic CAR target gene in the human liver is *CYP2B6*, which shows an induced expression after exposure to phenobarbital (PB), and to agonist ligands such as CITCO (Honkakoski et al., 1998; Wang and Negishi, 2003). However, CAR is also an apparently constitutive transactivator, whose constitutive activity is inhibited by the inverse agonist ligands androstanol and androstenol (Qatanani and Moore, 2005).

In the present study, we demonstrate that ATF5 is an unexpectedly abundant liver-enriched transcription factor whose expression declines in dedifferentiated hepatoma cells and in cultured hepatocytes. Among the several potential roles in the liver we have found ATF5 activates the human *CYP2B6*, and cooperates with CAR in sustaining the hepatic-specific expression of this CYP in human hepatocytes and hepatoma cells. We

have also found that ATF5, and its target gene *CYP2B6,* are induced under endoplasmic reticulum stress conditions, pointing to an alternative mechanism to adapt CYP-mediated detoxification to specific harmful stress conditions.

METHODS

Chemicals

CITCO, PB, tunicamycin and glucosamine were purchased from Sigma (Madrid, Spain). Dimethyl sulfoxide (DMSO) was purchased from Merk Pharma (Mollet del Vallés, Spain). Primers for polymerase chain reaction were purchased from Invitrogen (Barcelona, Spain).

Cell culture

Human hepatoma cells (HepG2, Hep3B and Mz) were plated in Ham´s F-12/Leibovitz L-15 (1/1, v/v) supplemented with 6% foetal calf serum and culture to 60-70% confluence. Human hepatoma BC2 cells were cultured in a mixture of 75% minimal essential medium and 25% Medium 199, supplemented with 10% foetal bovine serum, 1 mg/ml bovine serum albumin, 0.7 μM insulin and hydrocortisone hemisuccinate, and maintained at confluence for 3 weeks. HeLa (human cervix carcinoma) and HEK293 (AdE1A-transformed human embryonic kidney) were grown in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% foetal calf serum and maintained as monolayer cultures. The culture medium for HEK293 cells was also supplemented with 3.5 g/liter of glucose.

Human hepatocytes were either isolated from liver biopsies (1-3 g) of patients undergoing liver surgery after an informed consent or from liver organ donations for transplantation. All the liver samples were from healthy areas of the liver and from donors who were not suspected of harboring any infectious disease and tested negative for human immunodeficiency virus and hepatitis. None of the patients or donors was habitual consumers of alcohol or other drugs. Samples were obtained in conformance to the rules of

the Hospital's Ethics Committee. A total of ten liver biopsies (four male and six female, aged between 2 and 79 years) were used. Hepatocytes were isolated using a two-step perfusion technique (Gomez-Lechon and Castell, 2000), and then seeded onto fibronectin-coated plastic dishes (3.5 μg/cm^2) at a density of 8×10^4 viable cells/cm^2. Hepatocytes were cultured at 37ºC in an atmosphere of 5% CO_2 in air using Ham's F-12/Williams (1:1) medium supplemented with 2% newborn calf serum, 50 mU/ml penicillin, 50 μg/ml streptomycin, 0.1% bovine serum albumin, 10^{-8} M insulin , 25 μg/ml transferrin, 0.1 μM sodium selenite, 65.5 μM ethanolamine, 7.2 μM linoleic acid, 17.5 mM glucose, 6.14 mM ascorbic acid, and 0.64 mM *N*-omega-nitro-L-arginine methyl ester. The medium was changed 1 h later to remove unattached hepatocytes. At 24 h, the cells were shifted to serum-free hormone-supplemented medium (10 nM dexamethasone and insulin).

Adenoviral vectors and infection

A recombinant adenovirus encoding human ATF5 was produced as follows: ATF5 mRNA was amplified from a human liver cDNA pool (n=12) with the Expand High Fidelity PCR System (Roche, Barcelona, Spain). Primers for ATF5, forward: 5'-GCA CGA ATT CTA CAG CC**A TG**T CAC T-3' and reverse: 5'-CAG AAG C**TT** CAC CCC TGC CCT TCT A-3', amplified a predicted 879-bp DNA fragment. The forward primer includes an EcoRI site, while the reverse primer includes a HindIII site (underlined). The PCR product was purified by agarose gel electrophoresis, double digested with EcoRI/HindIII, and directionally ligated into the adenoviral shuttle vector pAC/CMVpLpA, which was previously digested with the same restriction enzymes. The presence of the insert in the correct orientation was confirmed by restriction enzyme digestion. Sequence analysis of the ATF5 insert revealed a single base change with regards to published human ATF5 sequences. This mutation results in a single

amino acid substitution (P159L; NP_036200.2) located out of the known functional domains of the protein. The plasmid construct was cotransfected with the vector pJM17, containing the full-length E1 defective adenovirus-5 genome (dl309), into HEK293 cells by calcium phosphate/DNA coprecipitation. A homologous recombination between adenovirus sequences in both the shuttle vector pAC/CMV-pLpA and the pJM17 plasmid generates a genome of a packable size in which most of the adenovirus early region 1 is lacking, thus rendering the recombinant virus replication defective (Jover et al., 2001). The resulting virus (named Ad-ATF5) was plaque purified, expanded into a high concentration stock and titrated by plaque assay as previously described.

To generate a recombinant adenovirus for the expression of human CAR, a similar strategy was used. The primers used to amplify CAR cDNA from human liver were: forward 5´-CCA CCC CAA CAG TCG ACG TCA TG-3´ and reverse 5´-GGT CCA AGC TTT TTC CCA CTC C-3´ (SalI and HindIII underlined). This primer pair amplified a predicted 1139-bp fragment. The presence of the insert in the correct orientation was confirmed by restriction enzyme digestion and sequence analysis.

Cultured human hepatocytes (24 h) and HepG2 cells were infected with recombinant adenoviruses for 120 min at a multiplicity of infection (m.o.i) ranging from 6-60 plaque-forming units/cell. Thereafter, cells were washed and fresh medium was added. At 48h post-transfection, cells were analysed or directly frozen in liquid N_2.

Endoplasmic reticulum stress induction

For endoplasmic reticulum (ER) stress, HepG2 cells were incubated with either 5 µg/ml tunicamycin or vehicle, dimethyl sulfoxide (DMSO), in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 6% foetal calf serum. Alternatively, HepG2 cells

and human hepatocytes were incubated in amino acid-free Krebs-Ringer-Bicarbonate buffer (KRB; 117.8 mM NaCl; 4.6 mM KCl; 1.2 mM $KHPO_4$; 1.2 mM $Mg2SO_4.7H_2O$; 25 mM $NaHCO_3$; 10 mM Glucose and 1.25 mM $CaCl_2$). At each time, cells were collected and washed once with PBS 1X and then processed for mRNA and/or protein extraction as described below.

Purification and quantification of mRNA levels

Total cellular RNA was extracted using Trizol reagent (Invitrogen, Barcelona, Spain). Total RNAs from the 18 human tissues analysed in Figure 1A were purchased from Ambion (FirstChoice Human Total RNA Survey Panel). Reverse transcription was done using the M-MLV enzyme and 1 µg of total RNA following the manufacturer's instructions (Invitrogen, Barcelona, Spain). cDNA was diluted at 1/20 and 3µl were amplified with the rapid thermal cycler "Lightcycler" from Roche (Barcelona, Spain) in 15 µl of LightCycler FastStart DNA Master SYBR Green I (Roche, Barcelona, Spain), 0.3 µM of each primer, and with the optimum amount of $MgCl_2$ (3-5 mM) (Table 1). In parallel, we analysed the mRNA concentration of several human housekeeping genes: porphobilinogen deaminase (PBGD), beta actin (β-ACT) and Tata-Box binding protein (TBP) as internal controls for normalisation. After initial denaturing for 8 min at 95ºC, amplification was performed using optimised conditions for each primer set (Table 1). PCR amplicons were confirmed to be specific both by size (agarose gel electrophoresis) and melting curve analysis. The real-time monitoring of the PCR reaction, and the precise quantification of the products in the exponential phase of the amplification, were performed with the Lightcycler quantification software according to the manufacturer's recommendations.

Extraction of Protein and Immunoblotting

Nuclear extracts from cultured cells were prepared as described (Andrews and Faller, 1991). Protein concentrations were measured by the Bradford assay (Bio-Rad, Madrid, Spain). Cell proteins were resolved by SDS-PAGE on a 12% gel, and the separated proteins were transferred electrophoretically to PVDF membranes (Millipore, Madrid, Spain) using the Miniprotean II system (Bio-Rad, Madrid, Spain). Membranes were blocked overnight in PBS containing 5% skimmed-milk and were immunolabelled for 1 hr at room temperature with ATF5 antipeptide antiserum kindly provided by Dr. Angelastro (Angelastro et al., 2003) or from Imgenex (IMG-3026, Imgenex, USA), incubated overnight at 4ºC. Dilution of both antibodies was 1/500 in PBS containing 5% skimmed-milk. For detection, blots were washed and probed with secondary horseradish peroxidase (HRP)-conjugated antibody (Santa Cruz Biotechnology, USA), and were visualised on film via an enhanced chemiluminescence detection kit (ECL; Amersham, Barcelona, Spain). Equal loading was verified by Coomasie staining of the membranes.

Statistical Analysis

Each experiment was performed in several independent cell cultures as indicated. Each quantitative determination was done at least in duplicate. The results are expressed as the mean value ±SD. Statistical significance was calculated by the Student´s test.

DMD Fast Forward. Published on March 10, 2008 as DOI: 10.1124/dmd.107.019380
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RESULTS

ATF5 expression is abundant and enriched in adult human liver, but down-regulated in cultured human hepatocytes and hepatoma cell lines.

Firstly, we wanted to confirm previous evidence suggesting that ATF5 mRNA is efficiently expressed in liver tissue and to know whether ATF5 is a liver-enriched transcription factor. To this end, ATF5 mRNA was quantified in 18 human tissues by quantitative real time RT-PCR (Q-RT-PCR), and was compared with other well characterised liver-enriched transcription factors (LETF): HNF3γ, HNF4α and C/EBPα. The results depicted in Figure 1A demonstrated that ATF5 mRNA is detectable in all tissues, but the highest expression is found in the liver, where the mRNA level is at least 7-fold higher than in any other tissue examined. The expression profile of ATF5 was more hepatic specific than other well recognised LETF, such as HNF3γ, HNF4α or C/EBPα. ATF4, the closest ATF5 homologue, shows a ubiquitous expression profile that is very different from the typical liver-enriched regulators in general and from ATF5 in particular (Figure 1A). On the other hand, ATF5 mRNA showed a higher concentration than the other LETFs in absolute terms (Figure 1B), indicating that ATF5 is an unusually abundant liver-enriched transcription factor.

Secondly, we investigated the expression of ATF5 in different hepatic and non-hepatic cell models. Preliminary evidence of a down-regulation of ATF5 in *in vitro* cell models was obtained from a whole genome expression analysis. Human liver tissue, human primary cultured hepatocytes and HepG2 cells were analysed and compared by using Affymetrix human expression arrays (HG-U133). The results showed that ATF5 mRNA was down-regulated in human *in vitro* hepatic cell systems. The ATF5 mRNA level in cultured human hepatocytes (48h) was only a 20-30% of the tissue level. More

strikingly, HepG2 cells only displayed 2% of the liver content (Table 2). The expression of other ATF factors (ATF2, 3 and 4) did not show a similar profile and were not repressed in human *in vitro* hepatic cell systems (Table 2).

To further demonstrate the ATF5 differential expression, we performed a Q-RT-PCR analysis in the human liver and in several hepatic and non-hepatic cell models. The results confirmed previous evidence from the expression arrays, and demonstrated that ATF5 is greatly down-regulated in human hepatoma cells to levels as low as those found in non-hepatic cell lines (Figure 2). Cultured human hepatocytes (48 h) also presented a significantly decreased level, although not as pronounced as in cell lines (Figure 2).

This data collectively demonstrate that a high ATF5 expression is associated with the adult hepatic phenotype, and also support the hypothesis that ATF5 may be a key transcription factor for the maintenance of hepatocyte differentiation and/or maturation.

Adenovirus-mediated re-expression of ATF5 in HepG2 cells selectively re-activates *CYP2B6*.

To gain insight into the role of ATF5 in the liver, we developed an adenoviral vector for the expression of ATF5. The developed adenoviral vector was tested in dose-response experiments in human HepG2 hepatoma cells. Total ATF5 mRNA was analysed by Q-RT-PCR. The results demonstrated an efficient expression of ATF5 mRNA to levels above those found in the human liver (Figure 3A). Two different human ATF5 antiserums were used to check the concomitant expression of ATF5 protein in infected HepG2 cells. Immunoblotting analysis of nuclear extracts showed a dose-response expression of a protein with an apparent molecular weight around 36 kD after transfection with Ad-ATF5 (Figure 3B).

To further investigate the potential role of ATF5 in the liver function, HepG2 cells were transfected with increasing doses of Ad-ATF5, and the impact on the expression of characteristic hepatic genes was assessed by Q-RT-PCR. As a member of the ATF/CREB family, ATF5 could play a role in the regulation of genes involved in the response to cAMP signaling, such as *PEPCK* or *AldoB*, two typical hepatic genes for glucose/fructose metabolism. The expression analysis showed that ATF5 was indeed able to significantly induce these genes. Nonetheless, their response to ATF5 was rather weak (Figure 4), and the dose-response profile did not change when cells where treated with either glucagon or 8-Br-cAMP (data not shown).

The battery of characteristic hepatic genes analysed also included eight drug-metabolizing CYPs. The results showed that *CYP2B6* was specifically and robustly up-regulated by ATF5 (Figure 4), suggesting that this transcription factor could be involved in the expression and regulation of the drug-metabolizing *CYP2B6* in the liver. We also found a weak activation of *CYP2C9, 2C19* and *1A2* (up to 2-fold increase) but *CYP2E1*, *2D6*, *3A4* and *3A5* were not activated by ATF5 (data not shown).

ATF5 cooperates with CAR in the transcriptional activation of *CYP2B6* in human hepatoma cells.

The nuclear receptor CAR is one of the best characterized transcriptional activators of *CYP2B6* (Kawamoto et al., 1999). Therefore, we investigated a potential cooperation between ATF5 and CAR in the regulation of this gene.

HepG2 cells were cotransfected with recombinant adenoviruses for ATF5 and CAR, and their effect on CYP2B6 mRNA levels was assessed by real-time RT-PCR. Transduction with Ad-ATF5 caused an important dose-dependent activation, but transduction with Ad-CAR had a more limited activating effect on *CYP2B6*. However,

the cotransfection of ATF5 and CAR elicited an important additive induction, leading to a 15-20-fold increase in the mRNA levels of CYP2B6 (Figure 5).

ATF5 and CAR promote higher *CYP2B6* basal expression and induction by xenochemicals in cultured human hepatocytes.

Our findings in HepG2 cells suggest that ATF5 and CAR can cooperate in *CYP2B6* transcription activation. However, the mechanism of nuclear translocation of CAR in response to inducers is not functional in HepG2 cells, where CAR is already present in the nuclei of non-induced cells (Kawamoto et al., 1999). Therefore, we investigated the effects of ATF5 and CAR on *CYP2B6* in cultured human hepatocytes.

First, we analyzed the time-course expression of these genes during the initial stages of culture and found that the level of ATF5 and CAR progressively decreased from the early beginning of the culture (i.e. isolation) to up to 10-15% of the initial content at 23 h (Figure 6). This down-regulation, which is associated with the adaptation of hepatocytes to tissue disaggregation and culture conditions, is a common feature of many typical liver-enriched transcription factors. CYP2B6 mRNA was also decreased along with culture time (Figure 6) but the downregulation of ATF5 preceded the decrease in CYP2B6 mRNA (Figure 6), which further supports the notion that ATF5 is associated with the differentiated hepatic phenotype and is a key activator of *CYP2B6* basal expression in the human liver.

Transduction of cultured human hepatocytes with Ad-ATF5 caused a dose-dependent increase in CYP2B6 mRNA levels (Figure 7, top panel). The dose-response of *CYP2B6* to Ad-ATF5 was much more significant when a small amount of Ad-CAR (2 m.o.i.) was cotransfected (Figure 7, lower panel), which supports again that *CYP2B6* is a downstream target of ATF5. Noteworthy, transduction with Ad-CAR had also a dose-

DMD Fast Forward. Published on March 10, 2008 as DOI: 10.1124/dmd.107.019380
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dependent activating effect on *CYP2B6* (Figure 7, middle panel). These data demonstrate that the sole transfection of CAR activates *CYP2B6* in cultured human hepatocytes, and suggest that, in our experimental conditions, human CAR has a constitutive activity and can reach the nucleus of hepatocytes in the absence of added inducers.

Regarding the cooperation between ATF5 and CAR (Figure 8), our results in human hepatocytes demonstrate that combined expression of these two factors causes additive or synergistic transactivation of *CYP2B6* in human hepatocytes (depending on the dose), as it was previously observed in HepG2 cells.

We next analyzed if the combination of ATF5 and CAR has also a significant role in the induction of *CYP2B6* by xenochemicals. Our results, showed in Figure 8, demonstrate that the response of human hepatocytes to prototypical inducers such as PB or CITCO can be enhanced by the transfection of ATF5 and CAR. However, the relative fold-increase of CYP2B6 mRNA after PB or CITCO when ATF5 and CAR are overexpressed is not larger than in the non-transfected human hepatocytes (Figure 8). Therefore, we conclude that ATF5 and CAR play a role in sustaining a high basal *CYP2B6* expression, which translates in higher induced levels when CAR is further activated by xenochemicals.

ATF5 and CYP2B6 are induced in endoplasmic reticulum (ER) stress responses.

Sequence homology analysis shows that ATF4 is the closest homologue of ATF5. One of the most relevant roles of ATF4 is the sensing and response of multiple intracellular stress pathways. The basic mechanism of ATF4 activation under stress conditions is translational induction after the phosphorylation of the translation initiation factor eIF2α (Rutkowski and Kaufman, 2003). ATF4 induction leads, in turn, to the regulation of

many target genes involved in both amino acid metabolism and transport, and redox chemistry to cope with the ER stress. Translational induction of ATF4 involves the differential contribution of two upstream ORFs (uORFs) in the 5' leader of the ATF4 mRNA (Vattem and Wek, 2004).

We wondered whether ATF5 had also conserved uORFs in the 5' leader of its mRNA. Sequence analysis demonstrated that, similarly to ATF4, ATF5 had two conserved uORFs with very similar coding lengths and number of spacing nucleotides (Figure 9A), suggesting a potential similar translational mechanism for both ATF factors.

This finding led us to assess the possibility of post-transcriptional induction of ATF5 under stress stimuli. We analysed ATF5 mRNA and protein levels in HepG2 cells deprived of nutrients by incubation in amino acid-free Krebs-Ringer-Bicarbonate buffer. Figure 9B evidences that, despite no significant ATF5 mRNA changes, there was an important induction of ATF5 protein in human hepatoma cells. Our results also indicate that ATF5 may be also involved in ER stress responses in the liver which, in turn, should involve an induction of *CYP2B6*, its target gene.

We investigated the effect of several stress stimuli in the *CYP2B6* expression level and found that the incubation of HepG2 cells with amino acid-free Krebs-Ringer-Bicarbonate buffer and tunicamycin caused a time-dependent increase of the CYP2B6 mRNA level (Figure 9B), whereas other ER-stress inducing compounds, such as glucosamine, triggered a more modest activation (data not shown). Tunicamycin causes ER stress by inhibiting N-glycosylation of newly synthesised proteins. As a positive control, we measured the mRNA level of CHOP, a well characterised ER stress response gene. The up-regulation of *CHOP* was also observed under the different stress conditions, but with a different time-course profile (Figure 9B). This result demonstrates that, HepG2 cells develop an effective stress response which involves

CYP2B6 induction. To extrapolate these results into a more physiological condition, we performed a pilot experiment in cultured human hepatocytes. The results in Figure 9C show that aminoacid deprivation (48 h) can also trigger a stress response involving *CYP2B6* induction in human hepatocytes.

DISCUSION

The regulation of *CYP2B6* in the liver is an important issue as far as drug metabolism and xenochemical toxicity are concerned (Hodgson and Rose, 2007). However, most studies on *CYP2B6* regulation have focused on the mechanism responsible for the transcriptional induction mediated by xenochemicals, where the role of the nuclear receptor CAR has been very intensely investigated (Wang and Negishi, 2003). However, CAR does not operate alone in the transactivation of CYP genes. Several liver-enriched transcription factors, nuclear receptors and coactivators (HNF4α, PXR, RXR, GR, TR, VDR, LXR, SRC1, PGC1α, etc.) collaborate with CAR and establish an extensive crosstalk, controlling signaling pathways that regulate the homeostasis of bile acids, lipids, hormones, glucose, inflammation and vitamins, among others (Pascussi et al., 2007). In spite of that, the relevance of many of these transcriptional regulators in the control of human *CYP2B6* by CAR remains to be investigated, and very few studies have dealt with the tissue-specific regulation of *CYP2B6* in the liver.

Previous evidence from our laboratory suggests that the *CYP2B6* expression is controlled by HNF4α and C/EBPα (Jover et al., 1998; Jover et al., 2001), two important liver-enriched transcription factors. In the present study, we have shown for the first time that ATF5, another liver-enriched transcription factor, is able to activate *CYP2B6* in cooperation with CAR. Moreover, we have also demonstrated that ATF5 could play a role in modulating the *CYP2B6* expression levels under specific stress conditions, such as amino acid limitation or chemical-induced stress.

The potentiation of *CYP2B6* expression by ATF5 and CAR was demonstrated both in the absence and presence of *CYP2B6* inducers. CAR is mainly located in the cytoplasm and chemical inducers facilitate its translocation into the nuclear compartment where

CAR shows constitutive activity and binds to responsive elements in DNA (Baes et al., 1994; Honkakoski et al., 1998). In our experimental conditions and in absence of chemical inducers, the transfection of CAR activated *CYP2B6* in both HepG2 cells and cultured human hepatocytes. The mechanism of nuclear translocation of CAR in response to inducers is not functional in HepG2 cells, where CAR is already present in the nuclei of non-induced cells (Kawamoto et al., 1999). However, our results also support the notion that in the absence of added inducers a fraction of human CAR traffics and reaches the nucleus in cultured human hepatocytes, despite cytoplasmic retention is operative in this cell model (Pascussi et al., 2000). Thus, our results suggest a basal constitutive activity of human CAR on *CYP2B6*, which is in agreement with two recent studies where CAR was also expressed in human hepatocytes by means of viral vectors (Kamiyama et al., 2007; Stoner et al., 2007)

CYP2B6 induction by xenochemicals (PB and CITCO) was also potentiated by the co-infection of human hepatocytes with Ad-ATF5 and Ad-CAR, although the relative fold-increase was not augmented respect the non-induced transfected cells. Thus, ATF5 and CAR play a role in sustaining a high basal *CYP2B6* expression, which turns out into higher induced levels when CAR is further activated by xenochemicals.

The molecular mechanism by which ATF5 causes an increase in the CYP2B6 mRNA expression remains to be elucidated. There is no previous report demonstrating functional ATF/CREB-like elements in the *CYP2B6* regulatory sequences. However, in a recent study on *CYP2B6* promoter polymorphism, a putative ATF binding site at about -1.85 Kb was predicted by *in silico* analysis (Zukunft et al., 2005), which is in the vicinity of the -1.7 Kb PBREM for CAR. A direct effect of ATF5 on the *CYP2B6* gene could also take place through C/EBP response elements. ATF5 has been seen to interact

with C/EBPβ (Nishizawa and Nagata, 1992), and ATF4 with C/EBPγ and C/EBPα (Gombart et al., 2007). Moreover, several C/EBP family proteins regulate *CYP2B* genes (Luc et al., 1996; Jover et al., 1998; Cassel et al., 2000), and play important roles in the differentiation and development of a number of tissues, including the liver and intestine. A hallmark of bZIP proteins is that they extensively heterodimerise with each other both inside and outside individual families, therefore, one manner in which ATF5 could activate *CYP2B6* is through the formation of ATF5-C/EBP heterodimers, as well as through the binding to either C/EBP sites or ATF-C/EBP composite sites.

An alternative indirect mechanism for the cooperation between ATF5 and CAR in the transactivation of *CYP2B6* could involve an increase in CAR nuclear translocation promoted by ATF5. In this regard, it has been shown that transfection of the coactivator GRIP1 mediates ligand-independent nuclear translocation and activation of CAR (Min et al., 2002) However, three independent experiments using Ad-GFP-CAR in human hepatocytes have demonstrated that ATF5 does not visibly potentiate CAR translocation to nucleus (data not shown).

In this study, we have found that ATF5 could up-regulate the *CYP2B6* expression after particular stress stimuli, such as amino acid limitation and endoplasmic reticulum stress induced by tunycamicin. This is a new finding which points to a potential role of *CYP2B6* in stress conditions that could require detoxification of potentially harmful compounds, such as bile acids or bilirubin, or in chemical-induced liver stress. In this respect, it is important to emphasise that CAR activity has been shown to ameliorate the effects of hyperbilirubinemia, caloric restriction and toxic bile acids (Goodwin and Moore, 2004). Our results suggest that ATF5 could also participate in these processes through its cooperation with CAR and the transactivation of *CYP2B6* to respond to the

harmful stress induced by endobiotics/xenobiotics with the induction of detoxification genes.

We have shown that stress conditions cause an up-regulation of ATF5 protein and a concomitant increase in CYP2B6 mRNA. Therefore, it is feasible that the increase in CYP2B6 is mediated, at least partially, by ATF5. However, we can not rule out that other stress-activated transcription factors of the ATF/CREB family are also involved in this response. One of the most likely candidates is ATF4, which is also translationally activated in stress responses (Rutkowski and Kaufman, 2003). We have measured ATF4 expression and induction by stress stimuli and found that it is operative in our hepatic cell models (data not shown). However, it is important to remark that contrary to ATF5, ATF4 is neither a liver-enriched transcription factor (Figure 1) nor a transcriptional regulator associated with the hepatic phenotype (Table 2), which talks against a specific role of ATF4 in supporting the transcription of *CYP2B6* in hepatocytes. On the other hand, ATF2, 3 and 6 are also involved in stress responses and can likely bind to the same DNA response elements in target genes (Clerk and Sugden, 1997; Hai and Hartman, 2001). In summary, ATF5 may not be the only factor involved in *CYP2B6* activation in stress responses and a comprehensive study by gain/loss-of-function experiments to ascertain the specific role of the different ATF/CREB candidates will be needed.

Tissue-expression analysis of ATF5 revealed that the liver is the organ with the highest ATF5 mRNA level (10-fold higher than any other tissue), which indicates that ATF5 should be a liver-enriched transcription factor. However, ATF5 mRNA is also detected in most of the tissues examined, which suggests ATF5 may have both ubiquitous and liver-specific roles. In consonance, ATF/CREB proteins are involved in the control of

development and homeostasis in different cell types (Pati et al., 1999; Persengiev and Green, 2003; Al Sarraj et al., 2005; Angelastro et al., 2005). ATF5 shares the ability with other ATF/CREB family members to regulate the transcriptional response to intracellular cAMP through cAMP responsive elements (CRE), and a limited number of studies have suggested this possibility. For instance, ATF5 has been shown to be able to bind to CRE sites (Peters et al., 2001; Forgacs et al., 2005), and to repress cAMP-induced transcription and CRE-mediated expression (Pati et al., 1999; Angelastro et al., 2003; Forgacs et al., 2005). However, the role of ATF5 in the regulation of cAMP-mediated responses may also be tissue-dependent as ATF5 did not affect CRE-containing reporter genes in human hepatoma cells (Al Sarraj et al., 2005). We have observed a weak transactivation of CRE-containing genes (*AldoB* and *PEPCK*) after Ad-ATF5 transfection, suggesting that this transcription factor only plays a minor role in the control of cAMP responsive genes in the liver.

Our experimental evidence supports the notion that ATF5 is associated with hepatocyte differentiation and/or the maintenance of the adult hepatic phenotype. This observation contrasts with the described function of ATF5 in brain development where it blocks the differentiation of neuroprogenitor cells into neurons and glia, and must be downregulated to allow this process to occur (Angelastro et al., 2005; Mason et al., 2005). The different function of ATF5 in both the liver and nervous system could be explained by its different embryonic origin. Moreover, in Caco-2 cells, a model for enterocyte differentiation, ATF5 also shows a significant up-regulation when cells reach the differentiated condition (Peters et al., 2001); and in ATDC5 cells, a model for chondrocyte differentiation, ATF5 is once more associated with the differentiated state (Shinomura et al., 2006). Further investigation is needed to understand the specific

transcriptional environment that determines the role of ATF5 in tissue development and differentiation.

In conclusion, our findings uncover a new abundant liver-enriched transcription factor which is associated not only with the regulation of drug-metabolising *CYP2B6* in the liver, but also with the differentiated adult hepatic phenotype. Moreover, this transcription factor can cooperate with CAR and participate in the adaptation of the liver to harmful stress responses through the up-regulation of detoxifying *CYP2B6*. A potential contribution of ATF5 to the variability of the CYP2B6 expression in the human population remains to be investigated.

ACKNOWLEDGMENTS

We thank Dr. Celia Martinez for her collaboration in our preliminary studies on ATF5 and to Dr. Angelastro for kindly providing antiserum against ATF5. We would also like to acknowledge C. Guzman, D. Hernandez, G. Perez, C. Corchero and E. Belenchon for their expert technical assistance.

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FOOTNOTES

a) Financial support from the European Project PREDICTOMICS No. LSHB-CT-2004-504761 and CARCINOGENOMICS No. LSH-2005-1.2.3-1

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DMD Fast Forward. Published on March 10, 2008 as DOI: 10.1124/dmd.107.019380
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LEGENDS FOR FIGURES

Figure 1. *ATF5 mRNA is enriched and abundantly expressed in the liver*. **A)** Q-RT-PCR analysis of ATF5, HNF3γ, HNF4α, C/EBPα and ATF4 in 18 different human tissues, as described in materials and methods. Data represent the mean of triplicate amplifications and is expressed as a percentage of liver level. ATF5, HNF4 and HNF3γ mRNAs were enriched in the liver. The highest expression level of C/EBPα mRNA was observed in adipose tissue (three times above the liver) in agreement with its relevant function in this tissue. ATF4 shows a ubiquitous expression profile and can not be considered liver-enriched. **B)** Absolute mRNA concentrations of liver-enriched factors were determined by real-time Q-RT-PCR analysis in a reference human liver cDNA pool (n=12). Concentration values were obtained by interpolation in standard curves and expressed as molecules of target mRNA per molecule of the human housekeeping PBGD mRNA. ATF5 showed the highest absolute mRNA level. Data represent the mean ± S.D. from 6 independent PCR analyses.

Figure 2. *ATF5 expression in different hepatic and non-hepatic cell lines*. Quantitative real-time RT-PCR analysis of ATF5 mRNA in Liver (n=6), cultured human hepatocytes (CH, n=3), hepatoma cell lines (BC2, HepG2, Hep3B and Mz, n=3-4) and non-hepatic cell lines (HeLa and HEK293, n=2-3) after 48 h of culture. Data were expressed as percentage of a reference human liver pool sample and represent the mean ± S.D.

Figure 3. *Dose-dependent increase in the ATF5 expression after adenoviral transfection of HepG2 cells*. **A)** Hepatoma cells were transduced with increasing doses (6–60 m.o.i.) of Ad-ATF5, empty adenovirus (Ad-pAC) or adenovirus encoding GFP

(Ad-GFP), and 48 h later the mRNA concentration of ATF5 was measured by Q-RT-PCR. Data were expressed as percentage of a reference human liver pool sample and represent the mean ± SD of three independent experiments. **B)** Immunoblotting analysis of Ad-ATF5 infected HepG2 cells with two different ATF5 antiserum (Ab-1 from Angelastro et al. (2005); Ab-2 from Imgenex) demonstrated a dose-dependent increase in the expression of a ~36 kDa protein. Fifty µg of nuclear protein extracts were loaded per lane. Coomasie blue staining of membranes was performed to check for equal loading (not shown).

Figure 4. *Transcriptional activation of liver genes by ATF5*. HepG2 cells were transduced with increasing doses of Ad-ATF5 (6-60 m.o.i) and 48 h later the mRNA concentration of characteristic hepatic genes was measured by Q-RT-PCR analysis. The two cAMP-regulated genes, *PEPCK* and *AldoB*, showed a modest induction, while *CYP2B6* demonstrated a strong response. Data represent the mean ± S.D. from four-eight independent experiments: *PEPCK* (n=8), *AldoB* (n=6) and *CYP2B6* (n=4). *, $p < 0.05$; **, $p < 0.005$ and ***, $p < 0.001$.

Figure 5. *Cooperative transactivation of CYP2B6 by ATF5 and CAR in HepG2*. Cells were transduce with 6 (+) or 24 (++) m.o.i of Ad-ATF5, with 3 (+) or 15 (++) m.o.i of Ad-CAR or a combination of both adenovirus. Total RNA was extracted and Q-RT-PCR analysis was performed 48 h post-transduction. Data were expressed as percentage of a reference human liver pool sample. Bars represent the mean ± S.D. from five independent experiments. Co-infection with Ad-ATF5 and Ad-CAR resulted in higher CYP2B6 mRNA levels than those attained by separated infections.

Figure 6. *Time-course expression of ATF5, CYP2B6 and CAR in culture of human hepatocytes*. Hepatocytes were isolated and cultured for different times, as indicated. Total RNA was isolated and mRNA level determined by Q-RT-PCR. Data were expressed as percentage of the mRNA level in the hepatocyte suspension (before seeding, t=0) and represent the mean ± S.D. from five independent experiments. Sample-to-sample variations were normalized as described in material and methods.

Figure 7. *Transcriptional activation of CYP2B6 by ATF5 and CAR in human hepatocytes*. Cultured primary hepatocytes were transduced with increasing doses of Ad-ATF5 (upper panel), Ad-CAR (middle panel) or Ad-ATF5 plus a fixed dose of 2 m.o.i. Ad-CAR (lower panel). Total RNA was extracted and Q-RT-PCR analysis was performed 48 h post-transduction. Data were expressed as percentage of a reference human liver pool sample. Bars represent the mean ± S.D. from three independent experiments.

Figure 8. *Cooperative transactivation of CYP2B6 by ATF5 and CAR in human hepatocytes*. Primary culture cells (24h) were transduce with 6 (+) or 24 (++) m.o.i of Ad-ATF5, with 2 (+) or 8 (++) m.o.i of Ad-CAR or a combination of both adenovirus. After 24h of transduction cells were incubated in medium with 1mM phenobarbital, 500 nM CITCO or 0.05% DMSO (Control). Total RNA was extracted and Q-RT-PCR analysis was performed 48 h post-transduction. Data were expressed as percentage of a reference human liver pool sample. Bars represent the mean ± S.D. from three independent experiments.

Figure 9. *ATF5 and its target gene, CYP2B6, are up-regulated in cell stress responses*. **A)** Comparative analysis of ATF5 mRNA sequences from human (NM_012068.2) and mouse (NM_030693) reveals a similar two-uORF organization as described for human ATF4 (NM_182810). Each panel is drawn to scale. Dark-colored boxes represent the two uORFs. The open white-colored box overlapping the uORF2 is the main coding region. The number of nucleotides (nt) between uORF1 and uORF2 and the number of amino acids (aa) encoded by each of the uORFs are indicated. **B)** Q-RT-PCR time-course analysis of *ATF5*, *CYP2B6* and *CHOP* in HepG2 cells incubated for 48 h with either Kreb-Ringer-Bicarbonate buffer (square), tunicamycin (5 µg/ml) (triangle) or control (dash line). Bars represent the mean ± S.D. from three to four independent experiments. *, $p < 0.05$; **, $p < 0.005$ and ***, $p < 0.001$. Upper panel insert: Immunoblotting analysis of total protein extracts (50 µg/lane) from HepG2 cells incubated with Kreb-Ringer-Bicarbonate buffer (KRB) or control medium (CT) and detected with an anti-ATF5 antiserum (Angelastro et al., 2005). Data were expressed as percentage of a reference human liver pool sample. **C)** Q-RT-PCR time-course analysis of *CYP2B6* and *CHOP* in human primary hepatocytes incubated with Kreb-Ringer-Bicarbonate buffer. Data represent the average of two independent determinations from the same hepatocyte culture (HL-682).

DMD Fast Forward. Published on March 10, 2008 as DOI: 10.1124/dmd.107.019380
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Table 1

Oligonucleotides for Q-RT-PCR

mRNA Genbank nº	5´-bp	Primers sequence	Size (bp)	PCR conditions[a]
ATF5 NM_012068.2	1003 1190	UP: GAA GCG GGC AGA GGG TGA G DN: CAG AAG CTT CAC CCC TGC CCT TCT A	188	60,13,37,3
CYP2B6 NM_000767.4	983 1281	UP: GGG AGA TTG AAC AGG TGA TT DN: CTA AGG AGA AGG GGA TAA AAG	298	60,10,40,4
CAR NM_005122.2	619 981	UP: TGC TGC CTC TGG TCA CAC ACT T DN: TCA ATC TCA TCT CTC TGG GTA AC	363	60,22,34,5
PEPCK NM_002591.2	713 1207	UP: CTG GGC GAT GGG GAG TTT GTC A DN: GAT TGT GTT CTT CTG GAT GGT CTT G	495	60,30,40,4
ALDOB NM_000035.2	580 1155	UP: TGA GGA TTG CCG ACC AGT GT DN: CCC AGA AGA ACC CGT GTG AAC	576	60,15,40,3
HNF3γ NM_004497.2	1079 1219	UP: GGA CGC GCC CTA CAA CTT C DN: TTA AGC AAA GAG CGG GAA TAG A	141	60,12,40,3
HNF4α NM_00457.3	948 1203	UP: GCC TAC CTC AAA GCC ATC DN: GAC CCT CCC AGC AGC ATC TC	256	61,18,35,3
C/EBPα NM_001364.2	922 1352	UP: GTG GAG ACG CAG CAG AAG DN: TTC CAA GGC ACA AGG TTA TC	431	62,26,35,5
ATF4 NM_001675.2	864 1056	UP: TCC AGC AAA GCA CCG CAA CA DN: CCA CAG CCA GCC ATT CGG AG	212	60,22,34,5
PBGD NM_000190.3	189 461	UP: CGG AAG AAA ACA GCC CAA AGA DN: TGA AGC CAG GAG GAA GCA CAG T	273	60,15,40,3
β-ACTIN NM_001101.2	512 943	UP: CGT ACC ACT GGC ATC GTG AT DN: GTG TTG GCG TAC AGG TCT TTG	432	62,15,40,4
TBP NM_003194.3	884 1004	UP: AAA ATG GTG TGC ACA GGA GCC DN: CAC ATC ACA GCT CCC CAC CAT	121	59,10,36,3

[a] PCR amplification conditions are given in this order: Annealing temperature (ºC), Extension Time (seconds), number of cycles and $MgCl_2$ concentration (mM)

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Table 2

Gene expression analysis of different ATF factors from oligonucleotide array data

		ATF5 [204998_s_at]	**ATF5** [204999_s_at]	**ATF4** [200779_at]	**ATF3** [202672_s_at]	**ATF2** [205446_s_at]	**ATF2** [212984_at]
Liver tissue	Mean	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**
	SD	0.19	0.25	0.34	0.36	0.28	0.20
Hepatocytes	Mean	**0.31**	**0.20**	**1.73**	**1.60**	**1.68**	**1.02**
	SD	0.06	0.06	0.87	1.22	0.51	0.42
HepG2	Mean	**0.02**	**0.02**	**0.98**	**0.89**	**3.25**	**1.29**
	SD	0.00	0.00	0.12	0.18	0.18	0.35

Total RNA was isolated from different human liver samples (n=6), cultured human hepatocytes (48 h culture) (n=3) and human hepatoma HepG2 cells (70% confluence) (n=3). RNA integrity was assessed by using an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). cRNAs were prepared according to one-cycle target labeling protocol starting from 5 μg of total RNA and hybridized to HG-U133 GeneChip oligonucleotide arrays (Affymetrix, Santa Clara, CA). After image analysis and array normalization, intensity data were expressed as fold-increase relative to liver tissue.

Figure 1

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A

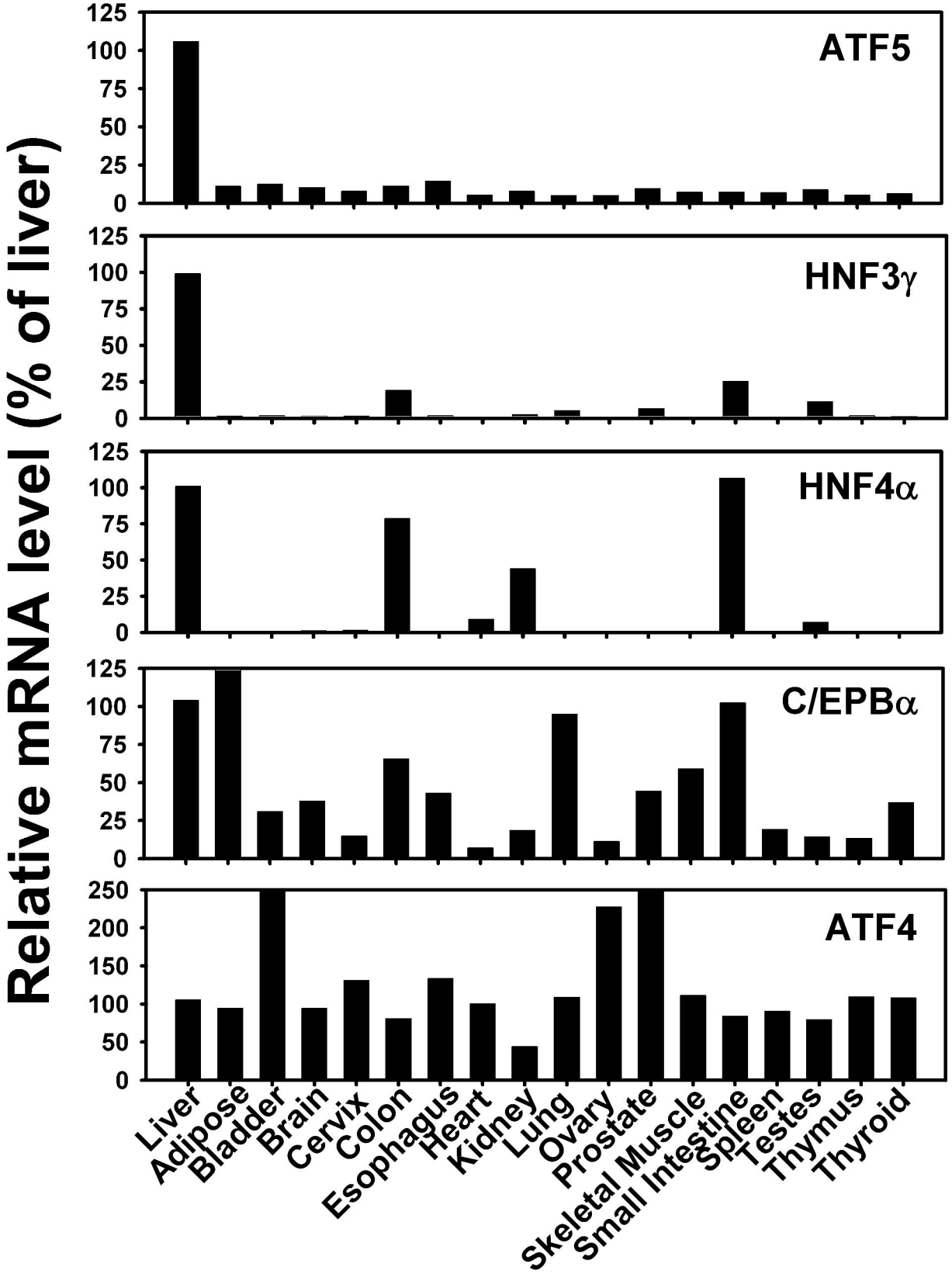

Figure 1

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B

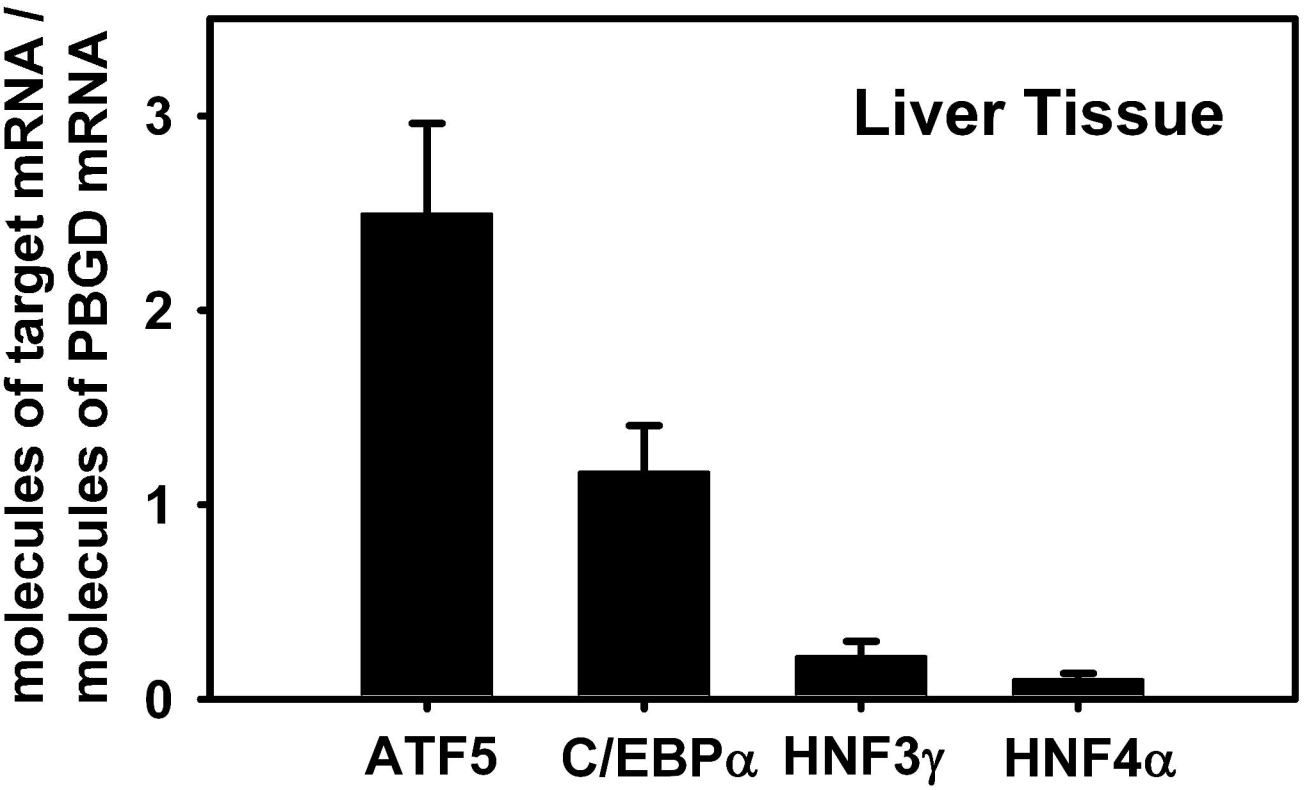

Figure 2

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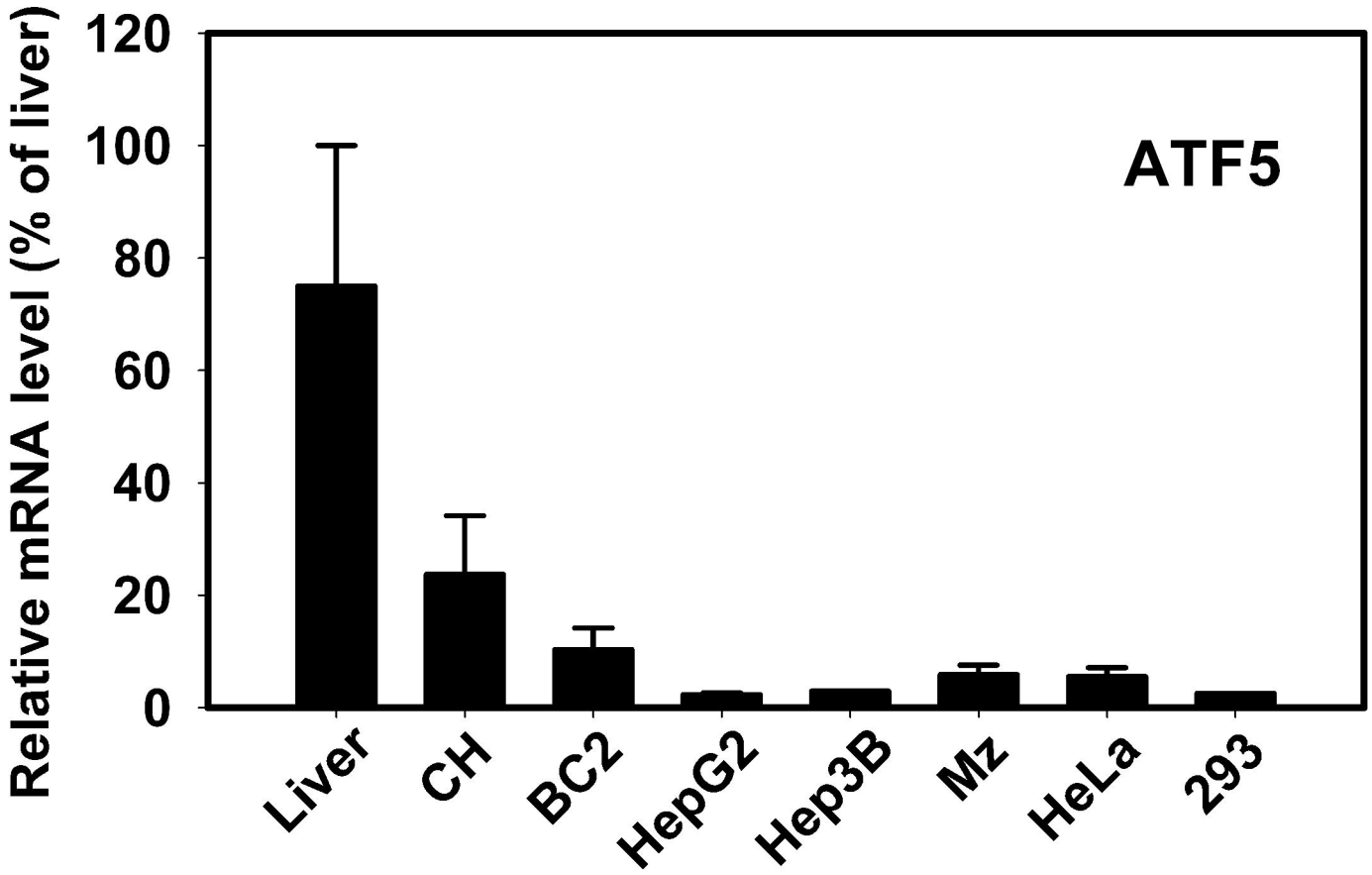


Figure 3

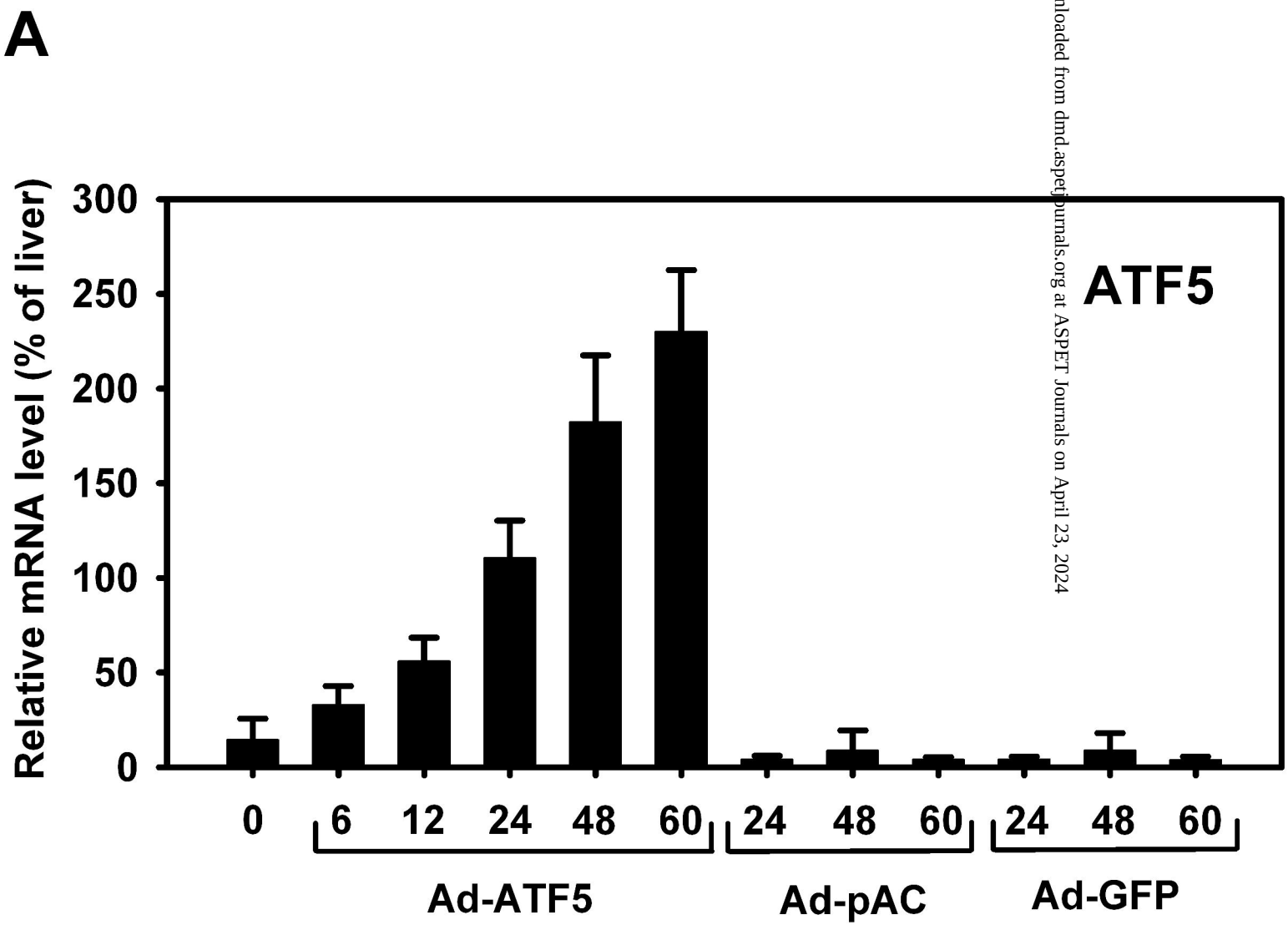
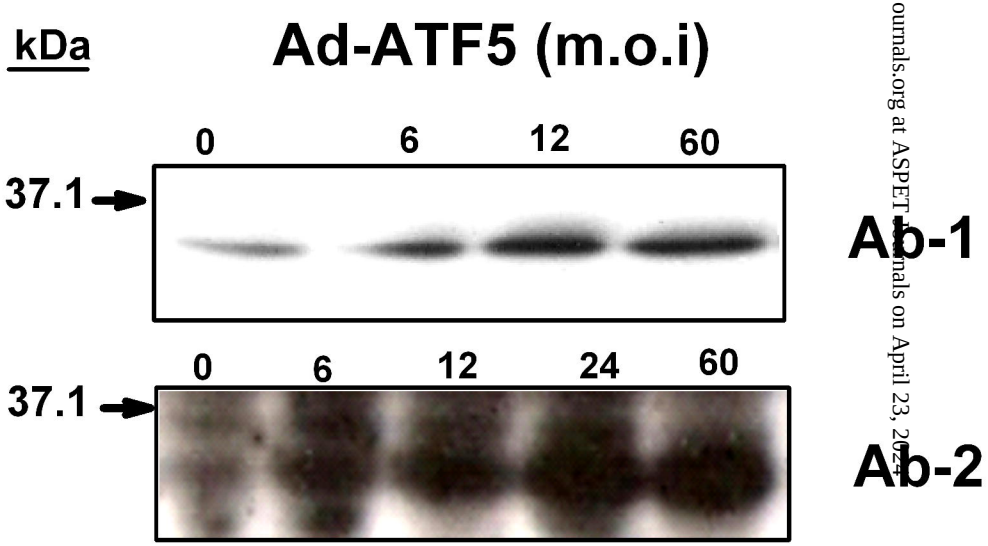


Figure 3

B



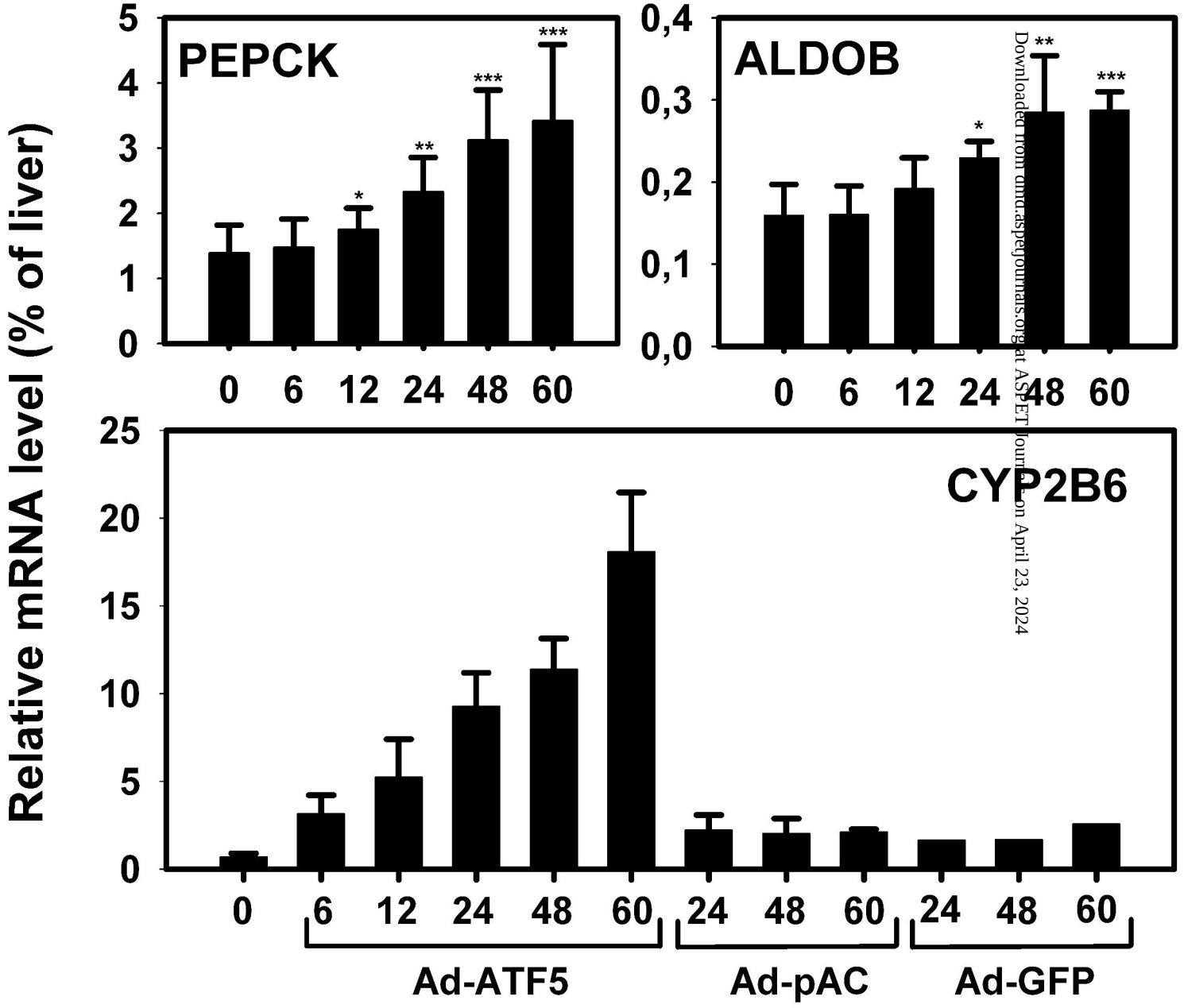


Figure 4

Figure 5

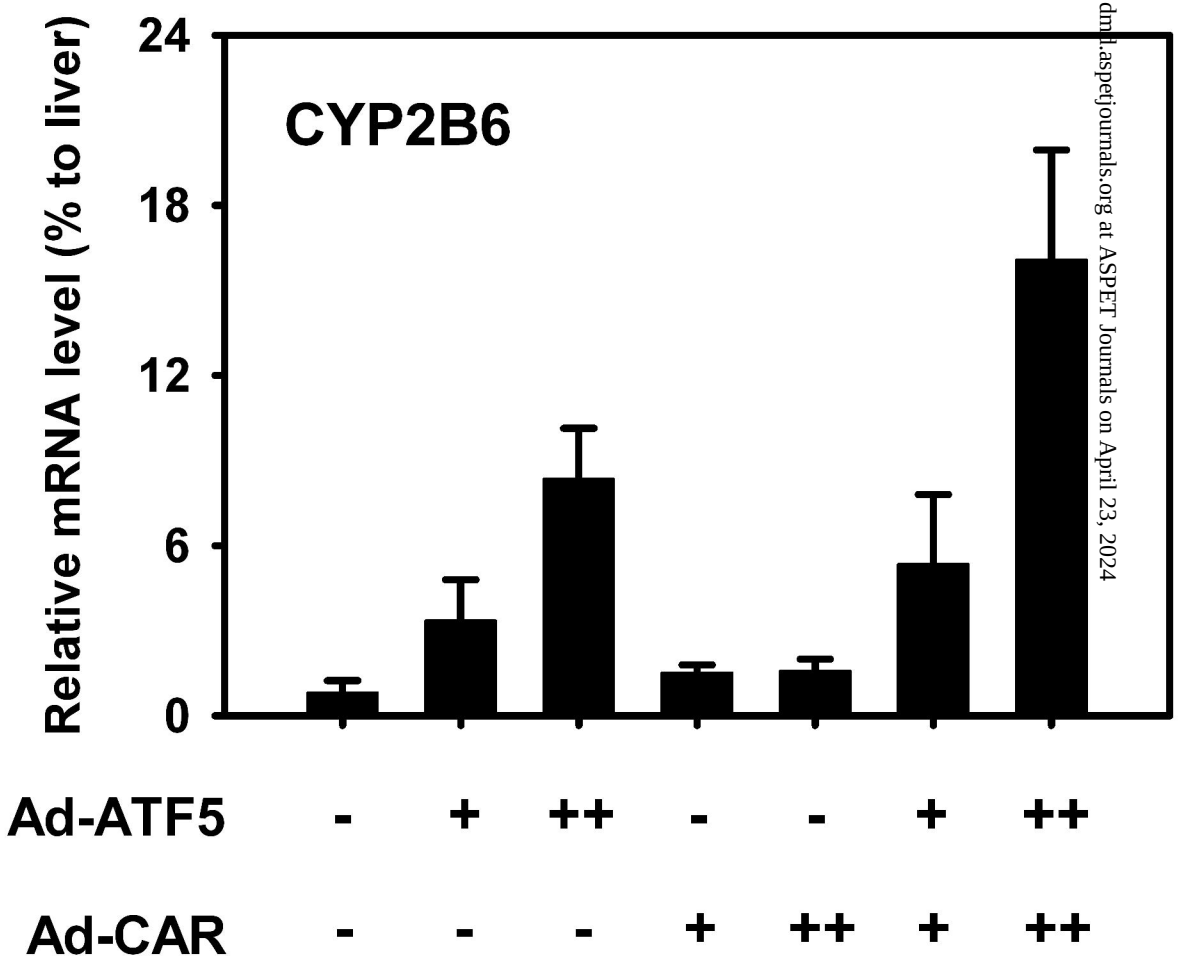


Figure 6

DMD Fast Forward. Published on March 22, 2007 as DOI: 10.1124/dmd.107.019380

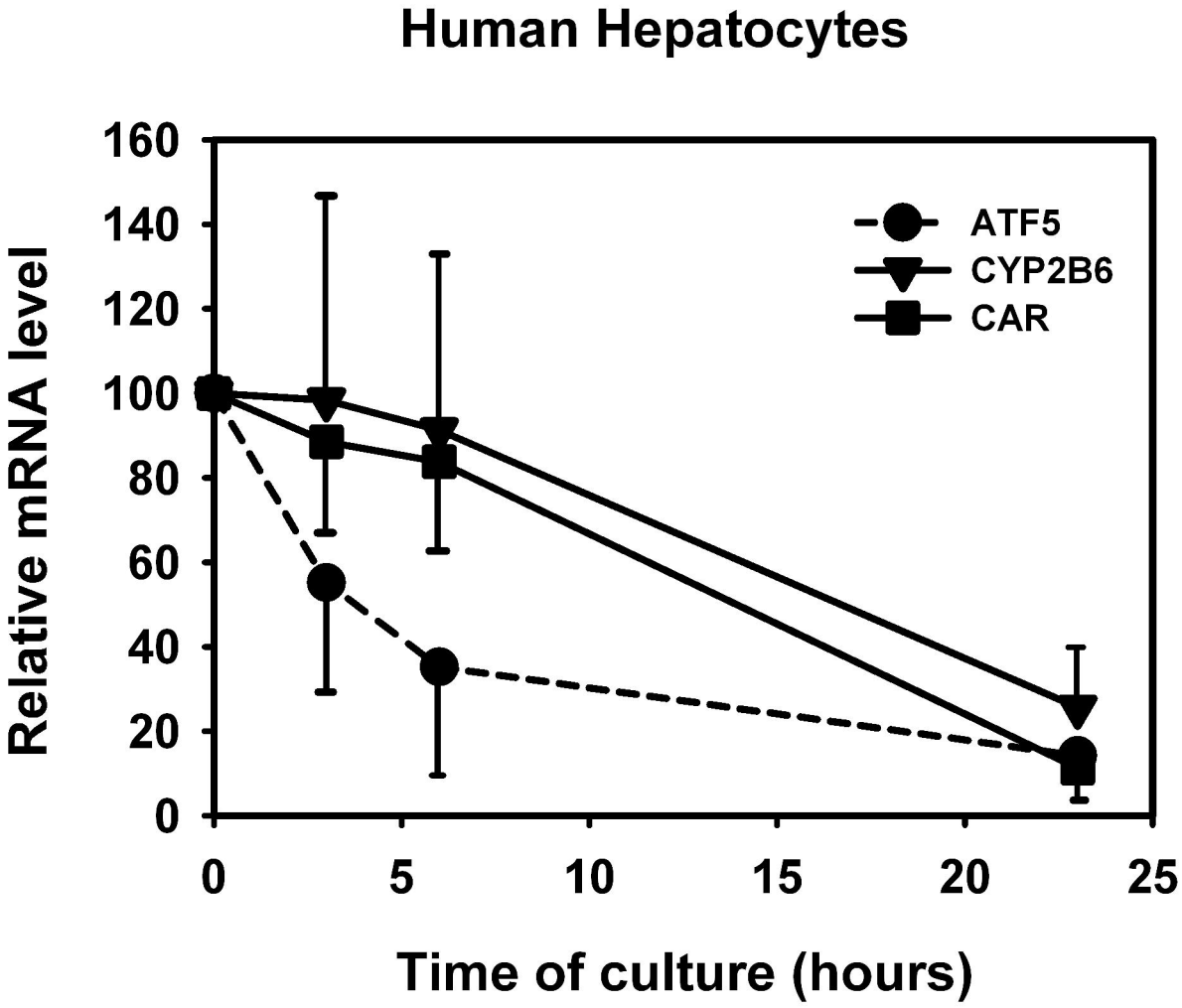


Figure 7

DMD Fast Forward. Published on March 10, 2008 as DOI: 10.1124/dmd.107.019380
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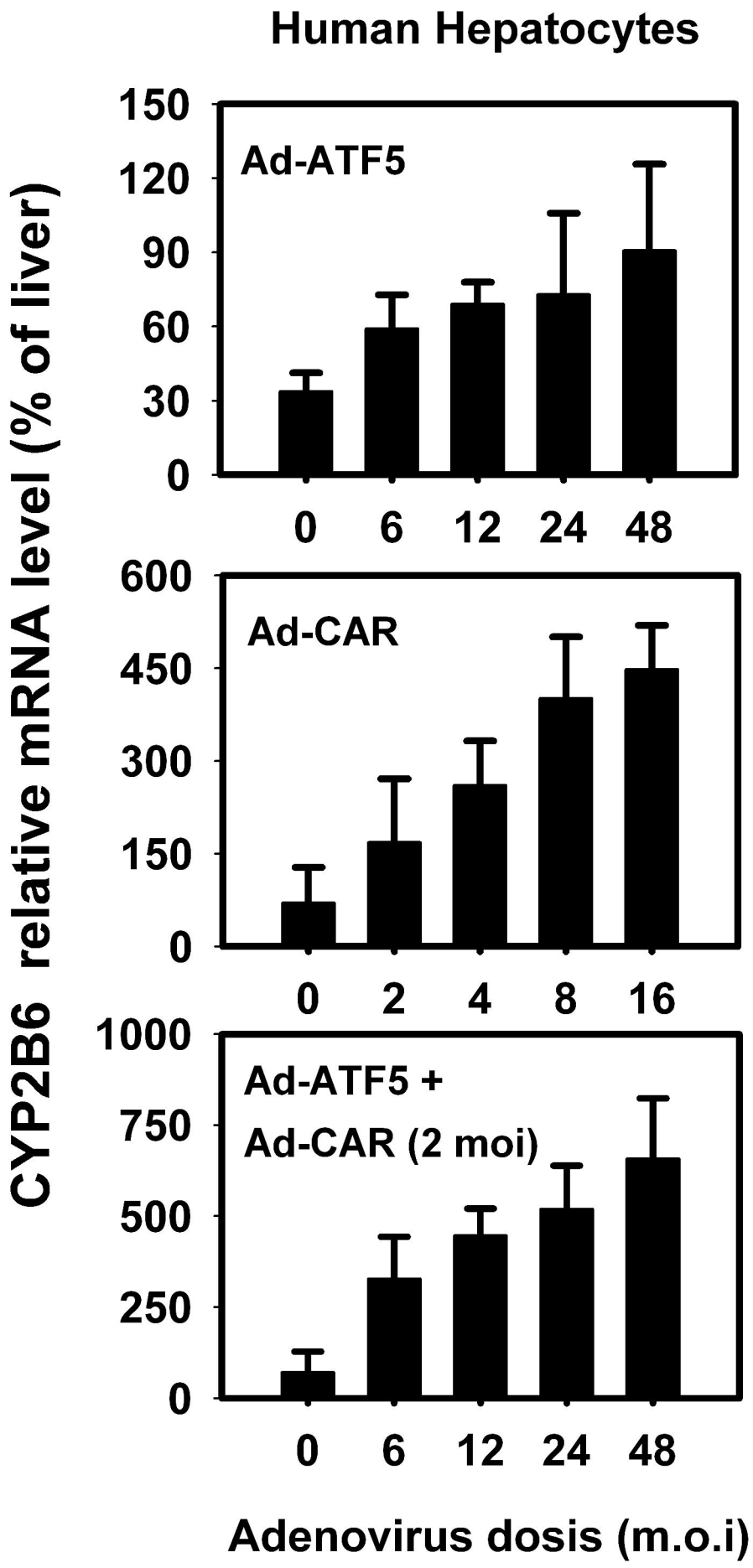

Figure 8

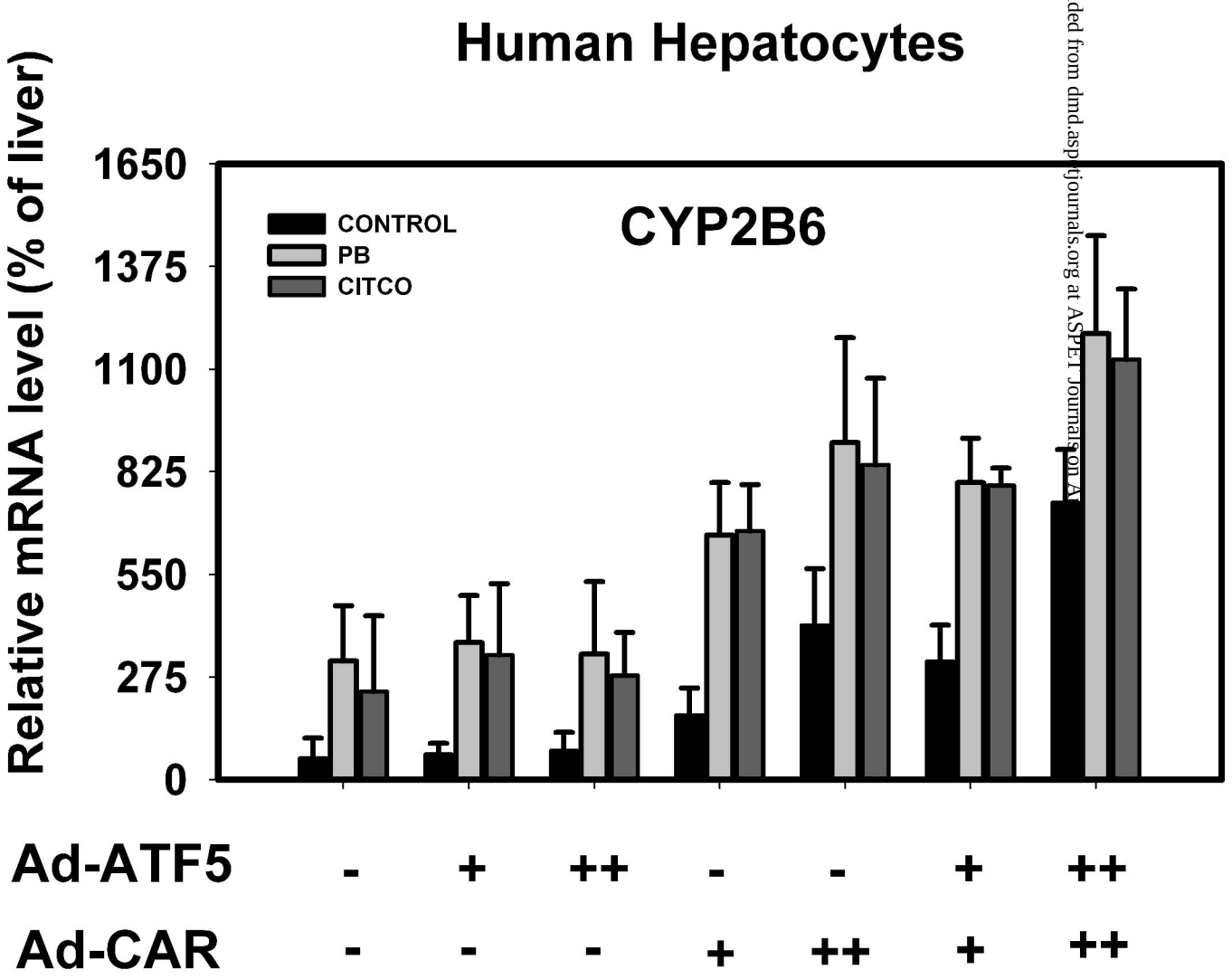

Figure 9

DMD Fast Forward. Published on March 10, 2008 as DOI: 10.1124/dmd.107.019380

A

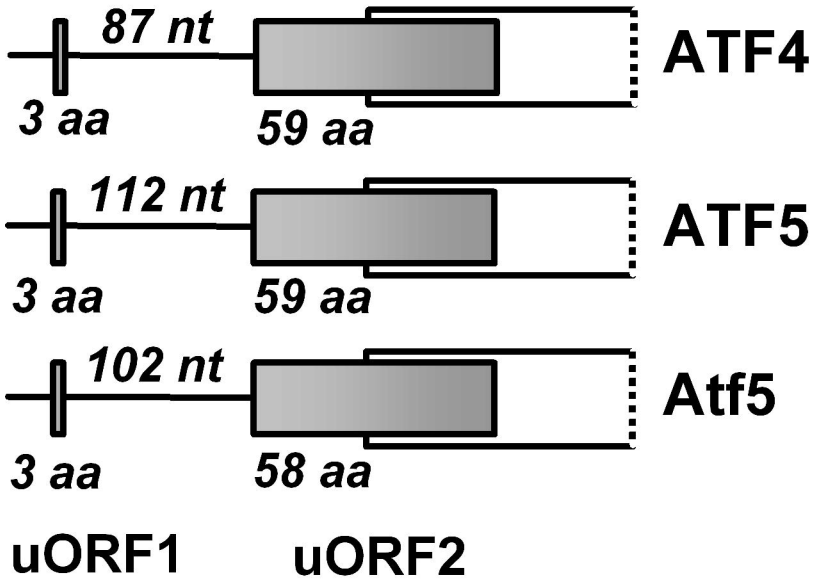

Figure 9

DMD Fast Forward. Published on March 10, 2008 as DOI: 10.1124/dmd.107.019380
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B

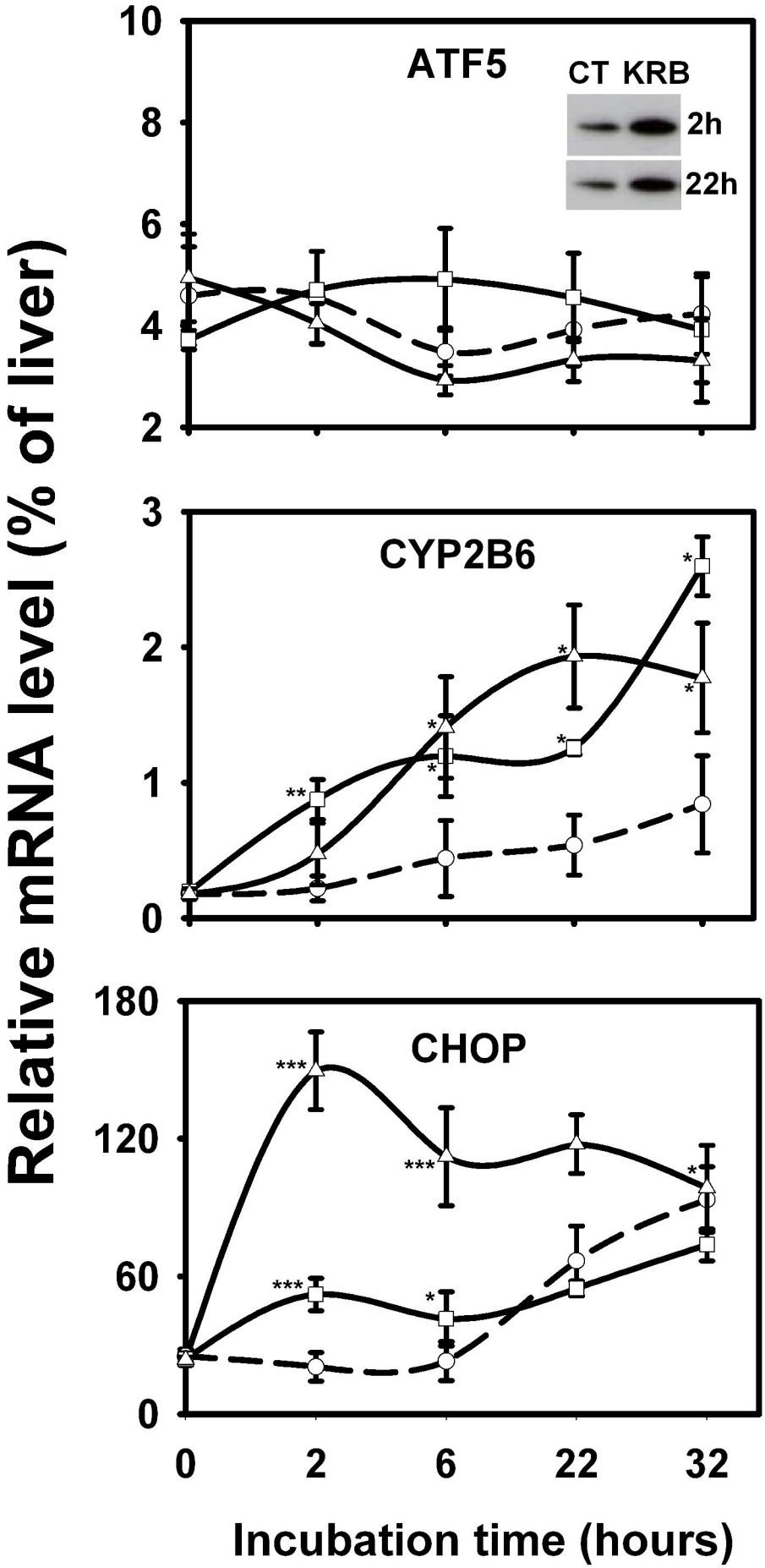

Figure 9

DMD Fast Forward. Published on March 10, 2008 as DOI: 10.1124/dmd.107.019380

C