One or More Labile Proteins Regulate the Stability of Chimeric mRNAs Containing the 3′-Untranslated Region of Cholesterol-7α-hydroxylase mRNA*

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Multiple AUUUA elements similar to those that regulate the degradation of several different mRNAs are conserved in the 3′-untranslated region (3′-UTR) of cholesterol-7α-hydroxylase (CYP7A1) mRNAs from several species. We examined if stabilization of mRNA decay could account for the >20-fold increase in the expression of CYP7A1 mRNA without a detectable change in transcription following dexamethasone treatment of rat hepatoma cells (L35 cells). Following RNA polymerase II-dependent transcription block or protein synthesis block, the decay of CYP7A1 mRNA displayed a short half-life (~30 min). Control experiments showed that in cells pre-treated with a RNA polymerase II inhibitor, dexamethasone had no detectable effect on CYP7A1 mRNA decay. Stable expression of luciferase reporter mRNAs in L35 cells showed that the CYP7A1 3′-UTR was required to observe a dexamethasone induction. To examine the hypothesis that a labile protein is required for dexamethasone-induced mRNA stabilization, cells were stably transfected with a tetracycline-repressible promoter that drives the expression of a green fluorescent protein analogue (ECFP) with or without the 3′-UTR of CYP7A1. Cells expressing ECFP with the 3′-UTR of CYP7A1 displayed a 30-fold dexamethasone induction of ECFP mRNA, whereas cells expressing ECFP without the 3′-UTR did not. Moreover, specific block of the transcription of ECFP containing the 3′-UTR by adding the tetracycline analogue doxycycline clearly displayed dexamethasone-induced stabilization of mRNA decay. These data provide compelling evidence that a putative labile protein and the 3′-UTR of CYP7A1 act together to decrease the rate of CYP7A1 mRNA degradation.

The initial step controlling bile acid synthesis from cholesterol is catalyzed by cholesterol-7α-hydroxylase (CYP7A11; EC 1.14.13.17) (reviewed in Refs. 1–5). The expression of CYP7A1 mRNA, protein, and enzyme activity varies rapidly and markedly in response to diurnal variation (6–8), dietary cholesterol (9–11), hormones (12–14), and cytokines (15). Changes in CYP7A1 gene transcription appear to play a major role in regulating expression levels (reviewed in Refs. 3–5). In cultured cells and rodents, several different DNA-binding proteins have been shown to regulate the transcription of the endogenous CYP7A1 gene in regard to diurnal variation (albumin D site-binding protein) (7, 8), liver specificity (CYP7A1 promoter binding factor) (16), oxysterols (liver X receptorα) (11, 17), and bile acids (basic transcription element-binding protein) (18) and (farnesoid X receptor) (19). Transcriptional variation results in an almost concomitant change in CYP7A1 mRNA levels, suggesting that CYP7A1 mRNA displays rapid turnover (6–8). Additional studies have led to the conclusion that these rapid diurnal variations are due to regulated degradation of its mRNA and protein (20).

Previous studies suggested that a post-transcriptional mechanism (e.g. stabilization of mRNA) might have been responsible for a >20-fold increase in the steady-state levels of CYP7A1 mRNA in L35 rat hepatoma cells treated with dexamethasone (14). This hypothesis was based solely on the observation that no detectable change in CYP7A1 transcription was observed in nuclei prepared from control and dexamethasone-treated cells (14). In this report we examine the effect of dexamethasone on the stability and expression of rat CYP7A1 and chimeric mRNAs encoding luciferase or an analogue of green fluorescent protein (ECFP). Our results show that the 3′-UTR of rat CYP7A1 and a labile protein, which is rapidly depleted from cells whose transcription or translation is blocked, are sufficient to allow dexamethasone-induced stabilization of mRNA decay.

MATERIALS AND METHODS

All reagents used for biochemical techniques were purchased from Sigma, VWR, or Fisher. Restriction enzymes and enzymes for labeling cDNA probes were purchased from New England Biolabs and Roche Molecular Biochemicals. Plasmid pDNA5 encoding a cytomegalovirus promoter and a neomycin resistance gene (G418 resistance), was purchased from Invitrogen. A modified version of the TetOff expression system (21) was purchased from CLONTECH (Palo Alto CA). Cell culture medium was obtained from Life Technologies, Inc., and serum was obtained from Gemini. The cDNA probes used for hybridizations have been described elsewhere (14, 22, 23).

Cell Culture

L35 rat hepatoma cells were cultured in Dulbecco’s modified Eagle’s medium as described in detail (14, 22, 23). Cells were treated with dexamethasone (0.1 mM), 5,6-dichlorobenzimidazole (DRB), cycloheximide, or doxycycline-HCl at the concentrations indicated in the figure legends. Control cells received ethanol (vehicle) only.

RNA Isolation and Quantitation

Cells were harvested at the times indicated in the figure legends by removing the culture medium and adding guanidinium isothiocyanate...
(26) published data. The sequence analyzer (DuPont) was in total agreement with the sequence containing the CYP7A1 3'-UTR in either orientation was sequenced. Sequences of the mRNA were obtained from human (46), rabbit (47), hamster (48), mouse (49), and rat (26, 28).

Luciferase Reporter Plasmid Constructs

The pcDNA3-Luc plasmid was constructed by ligating the firefly luciferase reporter gene into the BamHI-XhoI site of pcDNA3. This construct was used as the backbone for the subsequent addition of the 3'-UTR of CYP7A1. The entire 3'-UTR of rat CYP7A1 was obtained from two individual pBSSK-7α-3'-UTR plasmids (kindly supplied by Dr. John Chiang, Department of Biochemistry and Molecular Pathology, Northeastern Ohio Universities College of Medicine). The CYP7A1 3'-UTR was excised from the pBSSK-7α-3'-UTR plasmids with NolI and BspI20I and ligated into pcDNA3-Luc that had been linearized with BspI20I to form the pcDNA3-7α-3'-5' construct. This plasmid (designated pcDNA3-luc-7α-3'-5') was sequenced and shown to contain sequences identical to the 3'-UTR in the reverse orientation. To construct the luciferase plasmid with the 3'-UTR in the correct orientation, (pcDNA3-luc-7α-3'-5') a 3'-UTR plasmid (kindly supplied by Dr. John Chiang, Department of Biochemistry and Molecular Pathology, Northeastern Ohio Universities College of Medicine) was sequenced and shown to contain sequences identical to the 3'-UTR in the reverse orientation. The CYP7A1 3'-UTR was excised from the pcDNA3-Luc plasmid with NolI and BspI20I and ligated into pcDNA3-Luc that had been linearized with BspI20I to form the pcDNA3-7α-3'-5' construct. This plasmid (designated pcDNA3-luc-7α-3'-5') was sequenced and shown to contain sequences identical to the 3'-UTR in the reverse orientation.

 Luciferase Constructs—L35 rat hepatoma cells were cultured until 70% confluence. Each expression plasmid pcDNA3-Luc 3'-UTR and the control plasmid pcDNA3-Luc was transfected into L35 cells using CaPO4 as described (27). Cells were selected for stable expression of the plasmid expressing neomycin resistance by culturing in G418 (400 μg/ml). Once selected, at least three single cell clones/transfection were isolated.

ECFP Constructs—L35 cells were transfected with the TetOff regulatory plasmid and selected for resistance to the neomycin analogue, G418 (400 μg/ml). Single cell clones were isolated using the limiting dilution method. Clones were then screened for the presence of a doxycycline-repressible expression of luciferase (produced by a transiently transfected pBIL expression plasmid). A single cell clone (L35ctTA-X) exhibiting high expression of luciferase in the absence of doxycycline and low expression of luciferase in the presence of doxycycline was used to obtain stable clones of cells expressing the ECFP expression plasmids, as described below.

RESULTS

The 3'-UTR of CYP7A1 mRNA Contains AUUUA Elements That Are Conserved among Several Species—It has been generally noted that the 3'-UTR of CYP7A1 contains multiple AUUUA elements (6, 26, 28). In an appropriate context, AUUUA sequences in the 3'-UTR of several mRNAs influence mRNA stability (29–33). We examined the cDNAs from rat, hamster, mouse, rabbit, and human CYP7A1 for conserved AUUUA elements (6, 26, 28). In an appropriate context, AUUUA sequences in the 3'-UTR of several mRNAs influence mRNA stability (29–33). We examined the cDNAs from rat, hamster, mouse, rabbit, and human CYP7A1 for conserved AUUUA elements (6, 26, 28). In an appropriate context, AUUUA sequences in the 3'-UTR of several mRNAs influence mRNA stability (29–33).
combined with one to three AUUUA motifs have been shown to regulate the stability of other mRNAs (e.g. c-fos (34, 35)). Rat CYP7A1 mRNA also contains four heptameric UAUUUA(U/A) sequences, which may regulate mRNA stability when present in the 3′-UTR as three copies (31). In addition, the rat CYP7A1 mRNA contains a perfect nonomeric UUAUUU(U/A) sequence, which by itself has been shown to cause rapid degradation (31, 33). The phylogenetic conservation of several of these AUUUA elements in the 3′-UTR of CYP7A1 mRNA raises the possibility that they may play an important physiologic role.

**The Degradation Rate CYP7A1 mRNA Is Similar to That of c-myc**—We examined the rate of CYP7A1 mRNA decay following inhibition of RNA polymerase II-dependent transcription by DRB (36) in L35 cells treated with or without dexamethasone (Fig. 2A). At 0 h before the addition of DRB, the expression of CYP7A1 mRNA by L35 cells treated with dexamethasone was 20-fold compared with untreated cells (Fig. 2A). Within 1 h of adding DRB, approximately 75% of the CYP7A1 mRNA was lost from both groups of L35 cells. After this time up until 4 h, there was no detectable loss of the remaining CYP7A1 mRNA. The rate of decay of CYP7A1 mRNA (half-life ≈ 30 min) was similar to that of c-myc mRNA (half-life ≈ 36 min), a cell cycle-specific gene product whose mRNA displays rapid turnover. Furthermore, using this experimental protocol, dexamethasone treatment did not significantly affect the rate of decay of either CYP7A1 or c-myc mRNA. In HepG2 cells, dexamethasone did not alter the rate of decay of human CYP7A1 following polymerase II inhibition (37). These data suggest that either dexamethasone did not affect the rate of turnover of CYP7A1 mRNA or that a factor that is required for dexamethasone-mediated stabilization of CYP7A1 mRNA was lost upon RNA polymerase II block by DRB.

**Inhibition of Protein Synthesis by Cycloheximide Causes Rapid Degradation of CYP7A mRNA**—To determine if translation is required for the rapid degradation of CYP7A1 mRNA, dexamethasone-induced L35 cells were treated with cycloheximide (0.05 mg/ml). Under the conditions and time course of these experiments, there was no evidence of cell toxicity as determined by trypan blue exclusion (data not shown). Within 1 h of adding cycloheximide CYP7A1 mRNA decreased (Fig. 3) in a manner that was similar to the decrease observed follow-
ing RNA polymerase II block with DRB (Fig. 2). The decay of CYP7A1 mRNA was specific, since even after 4 h there was no detectable loss of mRNAs encoding β-actin (Fig. 3) or c-myc (data not shown). These data suggest that translation is not required for the rapid degradation of CYP7A1 mRNA and that a labile protein required for dexamethasone-mediated stabilization of CYP7A1 mRNA was lost upon cycloheximide treatment.

The 3′-UTR of the Rat CYP7A 3′-UTR Is Sufficient to Display Dexamethasone Induction of a Chimeric mRNA Encoding Luciferase—Cytomegalovirus promoter-driven expression plasmids encoding both a neomycin-resistant gene product and the enzyme luciferase were constructed so that the entire rat CYP7A1 3′-UTR was either absent or present 3′ to the luciferase mRNA. Single cell clones resistant to G418 were obtained and assayed for luciferase mRNA expression by Northern blotting (Fig. 4). Cells expressing the plasmid without the CYP7A1 3′-UTR contained a single luciferase mRNA (~2 kilobases) (Fig. 4). In the cells expressing luciferase containing the CYP7A1 3′-UTR, three different-sized mRNAs encoding luciferase were present (Fig. 4). These three luciferase mRNAs had sizes similar to the sizes of rat CYP7A1 mRNA plus an additional 0.16 kilobases, which is equal to the larger coding region of luciferase compared with CYP7A1. These data suggest that the different molecular weight forms of the rat CYP7A1 mRNA are produced by different usages of polyadenylation sites contained within the 3′-UTR, as predicted (28).

In the absence of dexamethasone, cells stably transfected with the luciferase plasmid without the 3′-UTR contained ~10-fold greater luciferase mRNA compared with cells stably transfected with the luciferase plasmid containing the 3′-UTR (Fig. 4). Moreover, dexamethasone caused a 3-fold increase in luciferase mRNA levels in 24 h but had no effect on the expression of luciferase without the 3′-UTR (Fig. 4). Similar results were obtained in a total of three separate single cell clones (i.e. dexamethasone treatment caused a 2-3-fold increase in the expression of mRNA encoding luciferase with the 3′-UTR, whereas there was no significant change in the level of luciferase mRNA without the 3′-UTR).

Additional experiments showed that dexamethasone did not affect the rate of degradation of the luciferase mRNA containing the 3′-UTR (i.e. following DRB-blocked transcription, the rate of decay was the similar in cells treated with and without dexamethasone; data not shown). These findings are similar to those observed for the endogenous CYP7A1 mRNA (i.e. dexamethasone increased steady-state mRNA levels without altering mRNA decay; Fig. 2). The inability to experimentally observe an effect of dexamethasone on the rate of turnover of mRNAs containing the CYP7A1 3′-UTR might be explained if dexamethasone mRNA stabilization required a labile protein that may have been depleted following transcription arrest.

A Labile Protein Is Required for Dexamethasone to Stabilize mRNA Containing the 3′-UTR of CYP7A1—To examine the hypothesis that a labile protein is necessary for dexamethasone-induced stabilization of mRNAs containing the CYP7A1 3′-UTR, we developed an experimental approach that would specifically block the transcription of a reporter mRNA with and without the CYP7A1 3′-UTR. A modified version of the TetOff expression system (21) (CLONTECH) was chosen for these experiments. This regulated mammalian expression plasmid system utilizes two plasmids, the TetOff regulator plasmid and the TRE response plasmid. The TetOff plasmid constitutively expresses a doxycycline-controlled transactivator (tTA), a fusion of the wild-type Tet repressor from Escherichia coli to the activation domain of VP16 from herpes simplex virus. A single cell clone of L35 cells stably expressing tTA was obtained and subsequently transfected with a response plasmid (pBI-L) that expressed either ECFP, or ECFP without the CYP7A1 3′-UTR. In addition, luciferase was also expressed from the bidirectional tTA response element.

The following characteristics were consistently observed in three separate single cell clones of L35 cells stably expressing mRNAs encoding ECFP with and without the CYP7A1 3′-UTR. In the absence of dexamethasone, cells expressing ECFP without the 3′-UTR displayed an easily visualized cyan fluorescence (Fig. 5). In marked contrast, cells expressing ECFP containing the 3′-UTR displayed a cyan fluorescence that was barely visible in the absence of dexamethasone (Fig. 5). The level of fluorescence displayed by the cells agreed with the relative level of expression of ECFP mRNA, as shown below. Similar to the results obtained with the luciferase constructs (Fig. 4), in the absence of dexamethasone, the expression of ECFP without the 3′-UTR was >10-fold that of ECFP containing the 3′-UTR. These data further support the conclusion that the 3′-UTR of CYP7A1 confers instability to chimeric mRNAs. Moreover, following treatment of cells with dexamethasone, the intensity of the fluorescence of the cells expressing ECFP without the 3′-UTR did not change, whereas the fluorescence of the cells expressing ECFP containing the 3′-UTR was significantly increased. Treating cells with the tetracycline analogue, doxycycline, decreased the fluorescence of both groups of cells whether
or not they were also treated with dexamethasone. These data show that the fluorescence of both groups of cells was blocked by doxycycline and that only the ECFP containing the 3'-UTR of CYP7A1 was increased in cells treated with dexamethasone.

Multiple mRNAs encoding ECFP containing the 3'-UTR (Fig. 6) corresponded in size to the multiple luciferase mRNAs containing the 3'-UTR (Fig. 4). Moreover, the level of fluorescence displayed by cells expressing ECFP mRNAs agreed closely with the levels of ECFP mRNA expression (Fig. 6). The level of ECFP mRNA containing the 3'-UTR was <10% of the level of mRNA encoding ECFP without the 3'-UTR. Moreover, dexamethasone caused a 3-fold increase in the relative content of mRNA encoding ECFP containing the 3'-UTR (Fig. 6). These data confirm the findings observed with the luciferase chimeras (i.e., the presence of the CYP7A1 3'-UTR is sufficient to confer dexamethasone induction).

To examine if dexamethasone affected the stability of the ECFP mRNA containing the 3'-UTR, plasmid-dependent transcription was specifically blocked by doxycycline. A rapid decrease in ECFP mRNA displayed a double-exponential decay (Fig. 6). The decrease in ECFP mRNA was specific as demonstrated by the observation that the relative abundance of other mRNAs (e.g., β-actin) was unchanged throughout the time course of the experiment (Fig. 6). Moreover, it is clear that dexamethasone treatment significantly decreased the rate of degradation of ECFP mRNA containing the 3'-UTR (Fig. 6). In cells treated with dexamethasone, the rate of decay of mRNA encoding ECFP containing the 3'-UTR was ∼4-fold slower than the rate of decay without dexamethasone treatment. In contrast, dexamethasone had no effect on the decay of luciferase mRNA (data not shown). These data provide compelling evidence that dexamethasone induces the expression of mRNAs containing the 3'-UTR of rat CYP7A1 by decreasing the rate of mRNA degradation.

**DISCUSSION**

Our results support the following conclusions. 1) In the absence of dexamethasone, the rate of degradation of rat CYP7A1 mRNA is relatively rapid (i.e., similar to that of c-myc) (Fig. 2); 2) in its natural context and when added to mRNAs encoding luciferase or ECFP, the rat CYP7A1 3'-UTR acts to decrease expression; 3) the steady-state levels of mRNAs encoding luciferase or ECFP that contain the 3'-UTR of CYP7A1 is increased in L35 cells treated with dexamethasone (Figs. 2, 4–6); 4) dexamethasone increases the levels of mRNA encoding ECFP containing the 3'-UTR of rat CYP7A1 by decreasing its rate of degradation; and 4) a labile protein, which is likely to be an RNA-binding protein, is required for dexamethasone stabilization of mRNAs containing the 3'-UTR of rat CYP7A1.

The Relative Rate of Degradation of CYP7A1 mRNA Is Rapid Due to Instability Elements in the 3'-UTR—In L35 cells, the rate of decay of CYP7A1 mRNA was similar to that of c-myc (Fig. 2), whose degradation is considered to be rapid (38). We estimated the half-life of CYP7A1 mRNA in L35 cells to be ∼30 min. This rate of endogenous CYP7A1 decay is similar to the rapid rate of decay in cells expressing ECFP containing the 3'-UTR and cultured without dexamethasone (half-life = 45.6 min, Fig. 6). In other experimental systems and treatments the half-life of CYP7A1 mRNA varied from 30 min (39) to ∼4 h (6, 37, 40). The combined data support the proposal that CYP7A1 mRNA displays a relatively rapid rate of degradation. Our additional finding that irrespective of dexamethasone pres-
ence, adding the CYP7A1 3′-UTR to mRNAs encoding both luciferase (Fig. 4) and ECFP (Fig. 6) resulted in markedly lower steady-state expression of these mRNAs. However, without the 3′-UTR there was >10-fold higher expression as compared with mRNAs containing the 3′-UTR. Assuming that the presence of 3′-UTR in the expression vector would not affect transcription, the increased steady-state levels of mRNAs without the CYP7A1 3′-UTR indicates that the 3′-UTR enhances mRNA degradation. These conclusions are consistent with the predictions suggesting that the 3′-UTR of CYP7A1 mRNAs acts to destabilize mRNA (6, 26, 28). It has been reported that the enzymatic activity produced by a chimeric mRNA containing the mouse CYP7A1 3′-UTR was significantly less than that produced by an mRNA without the 3′-UTR (41).

**The 3′-UTR of CYP7A1 Is Sufficient to Confer Dexamethasone Induction of mRNA Expression Levels**—The findings that mRNAs encoding either luciferase (Fig. 4) or ECFP (Fig. 6) displayed dexamethasone induction when they contained the 3′-UTR of rat CYP7A1, but no induction without the 3′-UTR, strongly indicate that non-coding sequences play a regulatory role in CYP7A1 expression. However, the 3-fold increase in luciferase (Fig. 4) and ECFP (Fig. 6) mRNA levels by dexamethasone is clearly less than the >20-fold induction of the endogenous CYP7A1 mRNA (Fig. 2). There are several possible explanations for this difference. First, dexamethasone might increase transcription of the endogenous CYP7A1 gene. Using nuclear extracts from L929 cells run-off transcription assays detected no significant increase with dexamethasone (14). An alternative possibility is that the coding region of CYP7A1, which also contains AUUUA elements (Fig. 1), may affect the dexamethasone-mediated stabilization. Finally, it is also possible that differences in the stoichiometric relationships between the mRNA and the factors that may decrease its degradation may account for this decreased induction. Our findings strongly support the conclusion that a labile protein(s) may be necessary for dexamethasone to decrease the degradation of an ECFP mRNA containing the 3′-UTR (Fig. 6). These data imply that the cellular content of this(these) protein(s) relative to the amount of mRNA containing the 3′-UTR of CYP7A1 plays a critical role in dexamethasone-induction of mRNA expression.

**A Labile Protein Is Required for Dexamethasone Stabilization of mRNAs Containing the 3′-UTR of Rat CYP7A1**—We were unable to directly measure a dexamethasone-induced stabilization of mRNA decay of either the endogenous CYP7A1 mRNA or the luciferase mRNA containing the 3′-UTR. However, using the tetracycline-regulatable expression vector, we were able to clearly detect a slower rate of degradation of the mRNA encoding ECFP with the CYP7A1 3′-UTR in dexamethasone-treated cells (Fig. 6). These data strongly support the conclusion that the 3′-UTR of CYP7A1 confers dexamethasone induction of mRNA expression by increasing mRNA stability. The requirement for a labile protein for dexamethasone stabilization of CYP7A1 mRNA can explain the unexpected finding that inhibition of protein synthesis by cycloheximide treatment decreased the cellular content of CYP7A1 mRNA (Fig. 3) at a rate that was similar to the decay rate caused by blocking transcription with DRB (Fig. 2).

**What Physiologic Functions Provided the Evolutionary Pressure to Conserve AUUUA Elements in the 3′-UTR of the CYP7A1 mRNA?**—Clearly, rapid and regulated changes in mRNA degradation coupled to changes in transcription afford a more immediate change in mRNA expression and, presumably, enzyme activity. Although, to our knowledge, our study is the first to demonstrate a regulated change in CYP7A1 mRNA degradation, there have been several reports providing data that indirectly predicted this possibility. Diurnal changes in CYP7A1 transcription are mediated by DBP, a diurnally regulated transcription factor that binds to 5′ sequences in the CYP7A1 promoter and activates transcription (7, 8). This transcriptional variation results in an almost concomitant change in CYP7A1 mRNA levels, leading to the conclusion that due to the presence of instability elements in the 3′-UTR, CYP7A1 mRNA displays rapid turnover (6–8, 20). Changes in CYP7A1 mRNA stability have been proposed to play a role in mediating bile acid repression of CYP7A1 transcription (7, 40–43). There are two secondary effects of CYP7A1 enzymatic action that may require rapid changes. 1) Bile acids are cytotoxic, and their synthesis may require rapid regulation to prevent excessive accumulation and 2) in the hepatocyte, control of the cellular pool of cholesterol is intimately linked to expression of CYP7A1. The relatively rapid and variable turnover rate of CYP7A1 mRNA may ensure that changes in transcription rapidly invoke changes in the functional expression of this physiologically important enzyme. Additional studies show that the 3′-UTR of rat CYP7A1 mRNA prevents the expression of CYP7A1 in several non-hepatic tissue culture cell lines (RAW 264.1 macrophages and Mc Ardle rat hepatoma cells). Removing the 3′-UTR of rat CYP7A1 results in a robust expression. These findings suggest that the factors necessary to stabilize rat CYP7A1 may contribute to its unique tissue (liver) and cell type (parenchymal cells located near effenter venules) (44, 45) expression.

Our combined data suggest that regulated degradation of CYP7A1 mRNA compliments the changes in CYP7A1 gene transcription to provide a rapid and complex adaptation of enzyme activity to the metabolic demands of the cell, liver and animal.

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