The 3′-untranslated region of the mouse cholesterol 7α-hydroxylase mRNA contains elements responsive to post-transcriptional regulation by bile acids

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To investigate the importance of the 3′-untranslated region (UTR) of the mouse cholesterol 7α-hydroxylase (cyp7) mRNA in post-transcriptional regulation of expression of the cyp7 gene, chimaeric genes encoding mRNA containing the structural sequence of chloramphenicol acetyltransferase (CAT) linked to either the 3′-UTR of the mouse cyp7 mRNA or the SV40 early gene mRNA were constructed. The human cytomegalovirus (CMV) promoter was used to drive the expression of all the chimaeric genes. Thus the transgenes had identical sequences in the promoter, the regions encoding the 5′-UTR and translated sequence but differed in the region encoding the 3′-UTR of their respective mRNA species. The transgene containing the entire cyp7 3′-UTR (designated CMV.CAT.CYP7) gave rise to CAT activity in transfected hepatoma cells that was one-quarter of that obtained in cells transfected with the transgene containing the SV40 3′-UTR (designated CMV.CAT.SV40). The 3′-UTR of the cyp7 mRNA contains sequences resembling AU-rich elements (AREs). Deleting eight of nine putative AREs from the CYP7 3′-UTR sequence increased the CAT activity to a level greater than that observed for CMV.CAT.SV40, whereas deletion of the intron region had no effect. These results show that the AREs of the 3′-UTR of the cyp7 mRNA decrease transgene expression. Bile acids are known to repress the expression of the cyp7 gene. To test whether the 3′-UTR of the cyp7 mRNA has a role in this process, the expression of the chimaeric genes was evaluated in hepatoma cells competent for bile acid uptake. Conjugated bile acids, but not unconjugated bile acids, further decreased the expression of the CMV.CAT.CYP7 transgene. The same bile acids had no effect on the expression of the CMV.CAT.SV40 transgene. Deletion of the intron from the cyp7 sequence did not alter the CAT activity compared with the parental plasmid, and also did not alter the sensitivity of the transgene to the conjugated bile acids. Deletion of the AREs from the cyp7 3′-UTR, which increased the expression of the transgene, did not abolish the sensitivity of the transgene to repression by conjugated bile acids. Thus the 3′-UTR of the mouse cyp7 mRNA also contains elements that facilitate the further repression of transgene expression in the presence of conjugated bile acids. The results indicate that the 3′-UTR of the mouse cyp7 mRNA contains information specifying regulation at the post-transcriptional level.

INTRODUCTION

Conversion of cholesterol into bile acids represents an important pathway by which cholesterol is eliminated from the body. Cholesterol 7α-hydroxylase (cyp7) initiates the conversion of cholesterol into bile acids in the liver (reviewed in [1]). Factors that influence the expression of the cyp7 gene can have a significant impact on the overall efficiency of bile acid biosynthesis and consequently on whole-body cholesterol metabolism (reviewed in [2]). It is known that the expression of the cyp7 gene is modulated by dietary factors. Dietary cholesterol or cholestyramine treatment has been shown to increase cyp7 mRNA abundance, whereas a diet supplemented with bile acids decreases cyp7 mRNA abundance [3–8]. Dietary fatty acids were recently shown to influence the regulatory potential of dietary cholesterol on the expression of the cyp7 gene [9]. It is not yet known whether cholesterol or bile acids regulate the expression of the cyp7 gene directly, or indirectly through an intermediary. It is generally accepted that the cyp7 mRNA has a short half-life [10–13] and therefore change in the rate of transcription of the cyp7 gene is a major parameter in determining the steady-state level of cyp7 mRNA. Nuclear run-on experiments revealed that bile acids decrease cyp7 mRNA abundance by an extent that cannot be fully accounted for by the decrease in transcription of the cyp7 gene [12,14] but subsequent studies have been unable to demonstrate a change in cyp7 mRNA turnover rate in response to bile acids [13,15]. Thus the regulation of expression of the cyp7 gene at the post-transcriptional level remains controversial. In this study we determined whether the 3′-untranslated region (UTR) of the cyp7 mRNA has a role in regulating the expression of the cyp7 gene.

EXPERIMENTAL

Mouse cyp7 genomic gene cloning and detection of cyp7 mRNA

A rat cyp7 cDNA fragment (nt 99–1541, numbering as in [3]), corresponding to the translated region of the cyp7 mRNA, was synthesized by DNA amplification *in vitro* with cDNA reverse-transcribed from rat liver poly(A)$^+$ RNA as templates. The cDNA fragment was digested with *Sst*I and the fragment corresponding to the proximal portion of the cDNA was used to screen a 129/J mouse genomic library. Clones obtained from this screen were counter-screened with the fragment corresponding to

Abbreviations used: ARE, AU-rich element; β-gal, β-galactosidase; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; cyp7, cholesterol 7α-hydroxylase; TCDCA, taurochenodeoxycholic acid; UTR, untranslated region.

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the distal portion of the cDNA probe. From this collection, one clone that contained the entire cyp7 gene was selected for detailed analysis. Restriction fragments containing sequences that hybridized to the rat cyp7 cDNA probe were subcloned and then sequenced with an Applied Biosystems 373A DNA sequencer, with primers based either on vector sequence or on determined 129/J mouse cyp7 gene sequences.

Liver poly(A)$^+$ RNA was isolated from C57BL/6 mice fed with chow, chow plus 2% (w/v) cholesterol or chow plus 2% (w/v) cholestyramine for 14 days. After fractionation by agarose gel electrophoresis, the RNA (2 μg) was transferred to a nylon membrane and probed with randomly ^{32}P-labelled rat cyp7 cDNA or with mouse genomic DNA fragments. Hybridization was done at 65 °C for 18 h with probes (approx. 10^8 c.p.m./μg) in buffer containing 0.25 M sodium phosphate (pH 7.2), 7% (w/v) SDS, 0.1% sodium pyrophosphate and 2 mM EDTA. Final washes of membranes were done at 65 °C with a solution containing 0.1 × SSC and 0.1% SDS. The hybridized RNA was detected by autoradiography.

Construction of gene chimaeras

The plasmid vector pBK-CMV (in which CMV stands for cytomegalovirus) (Stratagene, La Jolla, CA, U.S.A.) was used as the backbone to construct chimaeric genes. The region spanning the bacterial promoter and the multicloning site of pBK-CMV was replaced with a fragment containing a synthetic 5′-UTR plus the complete sequence coding for the bacterial chloramphenicol acetyltransferase (CAT), generated from pCAT-Basic (Promega Corporation, Madison, WI, U.S.A.) by DNA amplification *in vitro*. The resulting plasmid was designated pCMV.CAT.SV40 and was used as the reference plasmid in the transfection experiments. To construct chimaeric genes containing sequences corresponding to the 3′-UTR of the mouse cyp7 mRNA, the fragment containing the SV40 sequence was entirely excised from pCMV.CAT.SV40. A fragment of the mouse cyp7 gene (a *Bam*HI/*Eco*RI fragment containing part of exon 5, the entire intron 5 and the entire exon 6) was directionally inserted downstream of the CAT gene sequence (see Figure 1). The resulting plasmid was designated pCMV.CAT.CYP7. Because the CAT structural sequence contains a translation termination codon, the sequence in the chimaeric mRNA corresponding to the mouse cyp7 gene exons is not translated. Two derivatives of this plasmid were generated by deleting the *Mfe*I fragment [containing eight of nine AU-rich elements (AREs), nt 1801–3394] from exon 6 (giving rise to pCMV.CAT.CYP7.ΔARE), and by deleting the *Bam*HI to Asp-718 (at nt 1297) fragment containing the entire intron 5 (giving rise to pCMV.CAT.CYP7.ΔI) (see Figure 2 for nucleotide sequence).

Cell lines and transfection assays

McNtcp cells [16] were constructed by stably expressing the sodium taurocholate co-transporting polypeptide [17]. The chimaeric genes were introduced into McNtcp cells by $Ca_3(PO_4)_2$ co-precipitation. A plasmid encoding β-galactosidase (β-gal), also under the control of the CMV promoter, was co-transfected with the chimaeric genes as an internal reference standard. The bile acids were added to the culture medium of cells at 100 μM (unless otherwise noted) 24 h after the introduction of the plasmids. Extracts of transfected cells were prepared 48 h after the introduction of DNA, and assayed for both CAT and β-gal activities by standard methods. No large variations in β-gal activity between replicates or treatments were detected. Radioactivity of the CAT reaction products was quantified using a Fuji BAS1000 PhosphorImager.

RESULTS

The mouse gene and mRNA for cyp7

The cyp7 gene isolated from a 129/J mouse genomic library spans approx. 10 kb and is composed of six exons (Figure 1A). The size and organization of the mouse cyp7 gene resemble those of the rat, hamster and human cyp7 genes [18–21]. The sixth exon of the mouse cyp7 gene is the largest exon and encodes the entire 3′-UTR of the cyp7 mRNA (Figure 2), a feature that is consistent between all the known cyp7 genes [10,18–22]. The primary structure of the mouse cyp7 polypeptide predicted from the current and the previously reported mouse sequence [23] show slight differences (Gly-218, Phe-228, Arg-297 and Glu-403 compared with Ala-218, Leu-228, Ser-297 and Asp-403 respectively). Nevertheless there is a high degree of sequence identity (74%) between the amino acid sequences of the mouse, rat, hamster, and human cyp7 enzymes.

The number of cyp7 mRNA species detected by RNA blotting seems to vary with species. In human and hamster liver RNA, a single ~ 2.9 and ~ 3.1 kb cyp7 mRNA respectively is detectable [20,24]. In rat liver, a variable number of cyp7 mRNA species, ranging in size from ~ 1.9 to ~ 4.4 kb, have been reported [3,4,10]. In mice fed with a chow diet, a cyp7 mRNA of ~ 4 kb is detectable (Figure 3). Feeding mice with a chow diet supplemented with either cholesterol or cholestyramine increased the abundance of the ~ 4 kb cyp7 mRNA, and generated at least three additional species of cyp7 mRNA ranging in size from ~ 2.2 to ~ 6 kb. The various isoforms might also exist in the

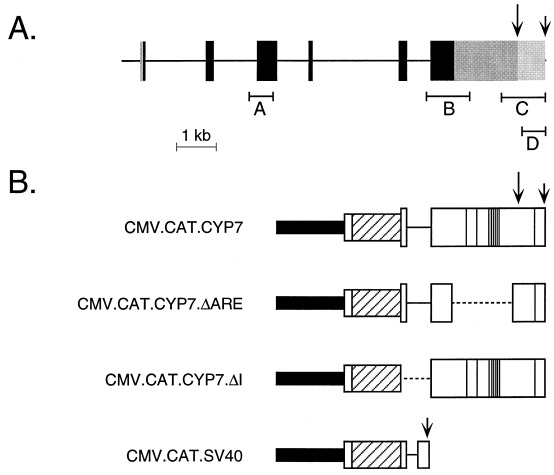

Figure 1 Structure of the mouse cyp7 gene and CAT gene chimaeras

(**A**) Organization of the mouse cyp7 gene. The black boxes represent regions that encode the translated portion of the mouse cyp7 mRNA and the grey boxes represent the UTRs. The dark grey box represents the regions included in the major cyp7 mRNA isoform; the light grey box represents the additional sequence included in the largest isoform of the cyp7 mRNA. The large and small vertical arrows show the positions of the major and probably the most distal polyadenylation signals respectively of the mouse cyp7 gene. Bars labelled A–D indicate the origins of mouse cyp7 gene probes used in the RNA blot shown in Figure 3. (**B**) Filled boxes depict the human CMV promoter; open boxes depict the UTRs of the gene chimaeras; stippled boxes represent the CAT structural gene sequence. The positions of the polyadenylation signals are shown by vertical arrows. The broken lines represent the regions deleted from the cyp7 sequence. The bottom diagram shows the location of AREs (▥) within the *Bam*HI/*Eco*RI fragment of the mouse cyp7 gene containing the sequence of the entire 3′-UTR of the cyp7 mRNA.

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Figure 2 Primary structure of exon 6 of the mouse cyp7 gene

Numbering of nucleotides corresponds to the mRNA sequence. The translation termination codon is shown in bold capitals. Sequences that resemble the canonical polyadenylation signal are shown in underlined capitals. Sequences that encode AREs (5′-AUUUA-3′) are shown in underlined lower-case letters.

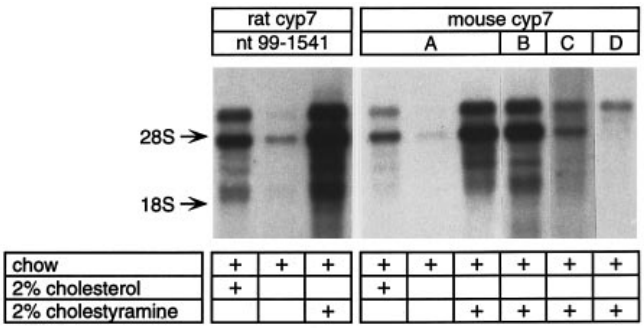

Figure 3 Detection of the mouse cyp7 mRNA

Liver poly(A)$^+$ RNA (2 μg per well) was analysed by RNA blotting. Multiple species of the cyp7 mRNA were detectable in the liver of mice fed the chow diet supplemented with 2% cholesterol or 2% cholestyramine. The origins of the mouse cyp7 gene probes (lanes A–D) are shown in Figure 1. Note that only liver poly(A)$^+$ RNA species from cholestyramine-fed mice were hybridized with probes B, C and D. The positions of the 28 S and 18 S ribosomal RNA size markers are shown at the left.

liver of chow-fed mice but are undetectable owing to extremely low abundance or rapid turnover.

Although multiple cyp7 mRNA species are detectable when the gene is induced by cholesterol and cholestyramine (Figure 3), relatively few sequences resembling the consensus polyadenylation signal are obvious in the 3′ region of the gene. However, it is likely that all the different cyp7 mRNA species contain a poly(A) tract because they are enriched in poly(A)$^+$ RNA preparations. To determine the sequence included in the major species of the mouse cyp7 mRNA (~ 4 kb), liver poly(A)$^+$ RNA was analysed by RNA blotting with probes derived from exon 6 of the mouse cyp7 gene (see Figure 1A). The most proximal 3′ probe (probe B, nt 1297–2272), which spans the first 695 nt of the 3′-UTR, detected the major species as well as several of the cyp7 mRNA isoforms. The major species plus the largest cyp7 mRNA isoform remained detectable with probe C (nt 3573–4320) but only the largest isoform was detected with probe D (nt 3965–4320). The results indicate that the major polyadenylation signal of the mouse cyp7 gene is located within probe C but upstream of probe D. Inspection of the sequence in this region revealed the existence of a variant polyadenylation signal (5′-ACTAAA-3′) upstream of probe D and situated 2154 nt downstream of the translation termination codon. Two hexanucleotide sequences (5′-AGTAAA-3′) that bear a resemblance to the canonical polyadenylation signal are also present within probe D. These sequences are within 2 nt of each other and 2606 nt downstream of the termination codon. The largest detectable isoform of the mouse cyp7 mRNA is probably polyadenylated under the control of either or both of these signals.

The 3′-UTR of the mouse cyp7 mRNA and structure of reporter gene chimaeras

The greatest degree of divergence in the primary sequences of cyp7 mRNA from different species occurs in the 3′-UTR of the cyp7 mRNA. In mice, the 3′-UTR accounts for more than 50% of the cyp7 mRNA. This region also contains numerous 5′-AUUUA-3′ repeats (Figure 2), a motif that is thought to be involved in regulating the stability of mRNA [25,26] as well as translational efficiency [27,28]. AREs exist in the 3′-UTR of the human [10], rat [22] and hamster [20] cyp7 mRNA species despite the sequence divergence, and might account for the short half-life of cyp7 mRNA *in vivo* [10–13].

To determine whether the 3′-UTR of the cyp7 mRNA has a role in regulating gene expression, chimaeric genes that encode mRNA bearing the CAT structural sequence plus the entire 3′-UTR of the mouse cyp7 mRNA (designated pCMV.CAT.CYP7) as well as two derivatives were constructed (Figure 1B). The pCMV.CAT.CYP7.ΔARE had eight of the nine AREs deleted and the pCMV.CAT.CYP7.ΔI had the cyp7 intron deleted. A chimaeric gene (designated pCMV.CAT.SV40) containing the region encoding the 3′-UTR of the SV40 early gene was used as the reference gene. All the gene chimaeras had identical 5′-UTR sequences and were under the control of the human CMV promoter. Thus the gene chimaeras differed only in the portion that encoded the 3′-UTR of their respective mRNA species.

Expression of the CMV.CAT.CYP7 transgene

The chimaeric genes were transiently expressed in McNtcp cells, a hepatoma cell line previously made competent to take up bile acids from the culture media [16]. The CAT activity of cells transfected with pCMV.CAT.CYP7 was one-quarter ($P < 0.0001$) that of cells transfected with pCMV.CAT.SV40 (Table 1). Cells transfected with pCMV.CAT.CYP7.ΔARE, which contains a deletion removing eight of the nine AREs from the cyp7 3′-UTR increased CAT activity to an extent that was greater than that observed in cells transfected with pCMV.CAT.SV40 ($P < 0.01$) (Table 1). The pCMV.CAT.CYP7.ΔI gave rise to CAT activity comparable to pCMV.CAT.CYP7 (the parental plasmid) (Table 1). Thus the decreased level of expression observed in cells

Table 1 Expression of the CAT gene chimaeras in McNtcp cells

CAT activity in McNtcp cells was assayed 36 h after the introduction of DNA. The transfection efficiency was normalized to the β-gal activity encoded by a co-transfected plasmid. Values shown are means $\pm$ S.D. for triplicate plates and expressed as percentages of the CAT activity encoded by the pCMV.CAT.SV40 plasmid (control). Significances of differences from the pCMV.CAT.SV40 control: $^{*}P < 0.0001$, $^{**}P < 0.01$ (Student's t test).

Transgene	Relative CAT activity (% of control)
CMV.CAT.SV40	100 $\pm$ 13
CMV.CAT.CYP7	23 $\pm$ 1*
CMV.CAT.CYP7.ΔARE	134 $\pm$ 14**
CMV.CAT.CYP7.ΔI	20 $\pm$ 3*

Table 2 Effect of bile acids on the expression of CMV.CAT.CYP7 and CMV.CAT.SV40 transgenes in McNtcp cells

McNtcp cells were transfected with the indicated plasmid DNA and the culture medium was replaced 12 h later with medium containing the indicated bile acids at 100 μM. CAT activity was assayed after an additional 24 h of incubation and normalized to the β-gal activity encoded by a co-transfected plasmid. Values shown (means $\pm$ S.D.; $n = 3$ plates) are expressed as percentages of the normalized CAT activity in the controls without bile acid for the respective sets. Similar results were obtained in replicate experiments. Significances of differences from the control without bile acid: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's t test).

Addition	Transgene expression (% of control)	
	CMV.CAT.CYP7	CMV.CAT.SV40
None	100 $\pm$ 6	100 $\pm$ 6
Taurocholic acid	81 $\pm$ 4**	101 $\pm$ 11
TCDCA	58 $\pm$ 9***	102 $\pm$ 8
Taurodeoxycholic acid	86 $\pm$ 4*	104 $\pm$ 10
Cholic acid	99 $\pm$ 8	99 $\pm$ 10
Chenodeoxycholic acid	106 $\pm$ 16	95 $\pm$ 8
Deoxycholic acid	110 $\pm$ 11	107 $\pm$ 13

transfected with pCMV.CAT.CYP7 is attributable to the presence of AREs in the 3′-UTR of the chimaeric mRNA.

Bile acid-mediated repression of transgene expression

To test whether the 3′-UTR of the cyp7 mRNA also has a role in repressing gene expression in response to bile acids, pCMV.CAT.CYP7 was transiently expressed in McNtcp cells in the absence or presence of unconjugated and taurine-conjugated forms of chenodeoxycholic (3α-, 7α-hydroxy), deoxycholic (3α-, 12α-hydroxy) and cholic (3α-, 7α-, 12α-hydroxy) acids. Addition of the bile acids to the culture medium at 100 μM did not significantly affect the viability of McNtcp cells (assessed by exclusion of Trypan Blue). This concentration of bile acids in the culture medium is in the physiological range and considerably lower than the critical micellar concentration. In general, addition of 100 μM conjugated bile acids to the culture medium consistently decreased the CAT activity by 20–40% in pCMV.CAT.CYP7-transfected cells ($P < 0.05$) (Table 2). Taurochenodeoxycholic acid (TCDCA) caused the greatest decrease in CAT activity ($P < 0.001$), whereas taurodeoxycholic acid caused the smallest decrease ($P < 0.05$). Addition of the corresponding unconjugated forms of the bile acids did not have the same negative effect on the CAT activity (Table 2).

Table 3 Effect of bile acids on CAT and β-gal enzyme activity

Bacterial cells expressing recombinant CAT or β-gal were washed and then lysed by sonication. The enzyme assay mixtures were supplemented with the indicated bile acid at 100 μM and then clarified bacterial lysate containing recombinant CAT or β-gal enzyme was added to initiate the reaction. Values shown are means $\pm$ S.D. for triplicate reactions and expressed as percentages of the respective controls (no addition).

Addition	Relative enzyme activity (% of control)	
	CAT	β-gal
None	100 $\pm$ 6	100 $\pm$ 6
Taurocholic acid	103 $\pm$ 4	101 $\pm$ 2
TCDCA	100 $\pm$ 8	99 $\pm$ 3
Taurodeoxycholic acid	103 $\pm$ 8	103 $\pm$ 2
Cholic acid	101 $\pm$ 9	96 $\pm$ 4
Chenodeoxycholic acid	100 $\pm$ 2	110 $\pm$ 6
Deoxycholic acid	103 $\pm$ 9	131 $\pm$ 18

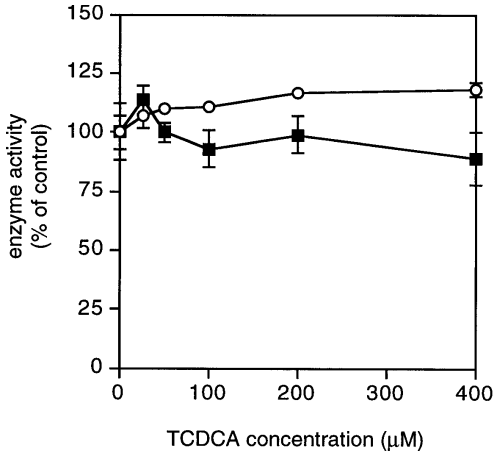

Figure 4 Effect of increasing TCDCA concentration on CAT and β-gal activities

The standard assay mixtures were supplemented with increasing concentrations of TCDCA, and clarified bacterial lysate containing recombinant CAT or β-gal was added to initiate the reaction. Values shown are means $\pm$ S.D. for triplicate reactions and expressed as percentages of the control without bile acid. Symbols: ■, CAT activity; ○, β-gal activity.

To determine the specificity of the bile acid-mediated repression of CMV.CAT.CYP7 transgene expression, the same series of bile acids were added to McNtcp cells that had been transfected with pCMV.CAT.SV40. No significant alteration in CAT activity was observed in the transfected cells after the addition of the conjugated or unconjugated bile acids to the culture medium, indicating that the CMV.CAT.SV40 transgene is insensitive to bile acids (Table 2). To confirm that the observed decrease in CAT activity in McNtcp cells was not due to a detergent effect on the enzyme itself, bile acids were added directly to the CAT reaction at a final concentration of 100 μM. As shown in Table 3, none of the bile acids tested had any appreciable effect on the activity of the CAT enzyme. In addition, increasing the TCDCA concentration did not significantly alter CAT activity even at the highest concentration used (400 μM) (Figure 4). These results indicate that the activity of the CMV promoter itself is not significantly altered by the presence of bile acids (see Table 2).

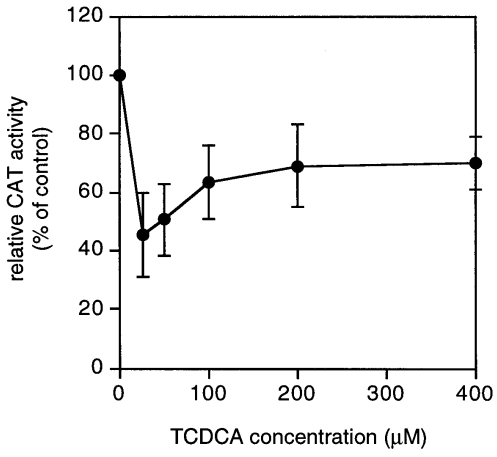

Figure 5 Bile acid concentration-dependent inhibition of CMV.CAT.CYP7 transgene expression

The medium of transfected McNtcp cells was replaced, 12 h after transfection, with medium containing increasing concentrations of TCDCA; CAT activity was assayed after a further 24 h. Values shown (means ± S.D.; $n = 3$ plates) are expressed as percentages of the control without bile acid.

The effect of bile acids on β-gal function was also tested because the β-gal activity encoded by a co-transfected plasmid was used to normalize the transfection efficiencies. There was little variation in the amount of β-gal activity in the extracts of transfected cells, regardless of the treatment condition. The activity of the β-gal enzyme itself was not affected by the different bile acids (Table 3), although a slight but not significant increase in β-gal activity was evident in response to increasing concentrations of TCDCA (Figure 4). Taken together, the results show that the 3′-UTR of the cyp7 mRNA contains elements that facilitate repression of transgene expression in response to conjugated bile acids.

Potency of TCDCA in repressing CMV.CAT.CYP7 transgene expression

The results from the previous experiments showed that the conjugated bile acids, but not the unconjugated forms, decreased the expression of CMV.CAT.CYP7 transgene. To determine the potency of conjugated bile acids in repressing the CMV.CAT.CYP7 transgene, the CAT activity in McNtcp cells transfected with pCMV.CAT.CYP7 was tested after the addition of increasing concentrations of a conjugated bile acid. TCDCA was chosen as the model conjugated bile acid because this bile acid showed the greatest degree of repression in previous experiments (see Table 2). As shown in Figure 5, expression of the CMV.CAT.CYP7 transgene was decreased when as little as 25 μM TCDCA was present in the culture medium. There was no evidence of cytotoxicity at this concentration but cell death was apparent when TCDCA was present in the culture medium at 400 μM.

AREs and bile acid response elements are distinct

The deletion derivatives of CMV.CAT.CYP7 were tested to determine whether the elements that facilitate the further repression of CMV.CAT.CYP7 transgene expression in response to the conjugated bile acids are co-localized with the AREs. In these experiments, TCDCA was added to the culture medium at 50 μM because near-maximal repression of CMV.CAT.CYP7 transgene expression was evident at this concentration. Chenodeoxycholic acid was also used to show that the inhibitory effect was caused specifically by conjugated forms of bile acids. The CAT activity in cells transfected with pCMV.CAT.CYP7.ΔARE was approx. 5-fold higher ($P < 0.01$) than in cells transfected with pCMV.CAT.CYP7, and the addition of TCDCA, but not chenodeoxycholic acid, to the culture medium still caused the decrease in transgene expression (Table 4). The same responses to the bile acids were observed in cells transfected with pCMV.CAT.CYP7.ΔI (Table 4), which contained the transgene lacking the cyp7 intron (see Figure 1). The magnitude of the decrease in the expression of the CAT.CYP7.ΔARE transgene (which contained only one of the eight AREs) was similar to that observed for CMV.CAT.CYP7 and CMV.CAT.CYP7.ΔI transgenes (i.e. approx. 40–50%). Thus the mechanism responsible for the bile acid-mediated decrease in transgene expression is most probably distinct from that mediated by AREs.

Table 4 Effect of bile acids on the expression of CMV.CAT.CYP7.ΔARE and CMV.CAT.CYP7.ΔI transgenes in McNtcp cells

McNtcp cells were transfected with pCMV.CAT.CYP7, pCMV.CAT.CYP7.ΔARE or pCMV.CAT.CYP7.ΔI along with a plasmid encoding β-gal. The culture medium was replaced 12 h later with medium containing no bile acid, 50 μM TCDCA or 50 μM chenodeoxycholic acid. CAT activity was determined after an additional 24 h incubation period. The CAT activity was normalized to the β-gal activity. Values shown are means ± S.D. for triplicate plates and expressed as percentages of the normalized CAT activity in extracts of cells transfected with the pCMV.CAT.CYP7 plasmid in the absence of bile acids. Significances of differences from the series control (without bile acid): $^*P < 0.001$ (Student's t test).

	Transgene expression (% of control)	
Addition	pCMV.CAT.CYP7.ΔARE	pCMV.CAT.CYP7.ΔI
None	575 ± 59	87 ± 12
TCDCA	367 ± 43*	45 ± 15*
Chenodeoxycholic acid	647 ± 49	93 ± 10

DISCUSSION

It is generally thought that expression of the cyp7 gene is regulated by bile acids via a classical negative feedback loop mechanism (reviewed in [1]), although several studies have yielded results that suggest the contrary [29–31]. Similarly, the role of cholesterol as a stimulator of expression of the cyp7 gene has been questioned. More recent data have emerged showing that dietary cholesterol has an inhibitory effect on the expression of the cyp7 gene in rhesus monkeys [32] and rabbits [33]. In mice, the ability of dietary cholesterol to stimulate or repress the expression of the cyp7 gene is dependent on the type of fatty acids present in the diet [9]. Experiments with isolated hepatocytes and some hepatoma cell lines have shown that the cyp7 gene responds as expected when bile acids or cholesterol are added to the culture medium [12,13,34,35]. However, it remains to be determined whether these factors act directly or indirectly in regulating the expression of the cyp7 gene.

The activity of the cyp7 enzyme is correlated with the abundance of the cyp7 mRNA [10–12,36], suggesting that cyp7 enzyme activity is controlled primarily at the pretranslational level. Ramirez et al. [6] proposed that the expression of the rat cyp7 gene was controlled primarily at the transcriptional level on the basis of results obtained from transgenic mouse studies. In contrast, Pandak et al. [14] have shown that different bile acid

species decrease the steady-state level of the cyp7 mRNA by different amounts, even though the inhibitions of transcription of the cyp7 gene assessed by run-on transcription were similar. This finding suggests that cyp7 mRNA abundance might also be regulated by post-transcriptional mechanisms.

Determination of the structure of the mouse cyp7 gene revealed that approx. 50% of the cyp7 mRNA is accounted for by untranslated sequences. Inspection of the sequence that encodes the 3′-UTR of the cyp7 mRNA revealed the existence of at least nine regions with sequences that resemble AREs, a motif that is commonly found in unstable mRNA species (reviewed in [37]). Despite the significant divergence in primary structure of 3′-UTRs of the mouse (the present study), rat and human cyp7 mRNA [4,10,24], AREs are a common feature in this region. The existence of the AREs in the 3′-UTR of the cyp7 mRNA is compatible with the observed rapid turnover of this mRNA *in vivo* [12,13].

The present study was conducted to assess the importance of the cyp7 3′-UTR in regulating the expression of the cyp7 gene independently of the mechanisms that regulate the transcription of the cyp7 gene, and revealed that the 3′-UTR of the mouse cyp7 mRNA contains information specifying regulation at the post-transcriptional level. Removal of the AREs from the cyp7 sequence resulted in a 5-fold increase in transgene expression, indicating that the AREs are functional. Deletion of AREs from the 3′-UTR of short-lived mRNA markedly improves stability (see [37]). The rapid turnover of the cyp7 mRNA *in vivo* is most probably due to the existence of AREs in its 3′-UTR. The possibility that the AREs also inhibit the translation of the cyp7 mRNA cannot yet be excluded.

Feeding studies show that different species of both conjugated and unconjugated bile acids decrease cyp7 mRNA abundance to different extents despite a similar inhibition of cyp7 gene transcription [14]. However, conflicting results have been reported regarding the regulation of cyp7 gene expression in hepatocytes and hepatoma cell lines. In these experiments, hydrophobic species of bile acids seem to have the greatest potency in inhibiting cyp7 gene expression [12,35,38], although this can be explained partly by the fact that cultured hepatocytes lose the ability to take up bile acids [16,39] and hydrophobic bile acids are most efficient in penetrating the cell membrane.

The present experiments were conducted with hepatoma cells that were intentionally made to take up bile acids from the culture medium [16]. Concentrations of less than 100 μM TCDCA in the medium were the most potent in inhibiting CMV.CAT.CYP7 gene expression in McNtcp cells. The greatest degree of inhibition was evident at 12.5 μM TCDCA. The exact basis for this observation is not clear. The mechanism responsible for inhibiting the expression of cyp7 3′-UTR-containing transgenes is most probably distinct from that mediated by AREs because the same inhibitory effect of bile acids was observed in cells transfected with pCMV.CAT.CYP7.ΔARE, which contains only one of the eight AREs. Otherwise the magnitude of the bile acid-mediated inhibition would be directly proportional to the number of AREs present in the 3′-UTR of the mRNA encoded by the transgenes.

Twisk et al. [12] previously demonstrated that both conjugated and unconjugated bile acid species inhibit the expression of the cyp7 gene in isolated rat hepatocytes. Unconjugated bile acids have a lower affinity for sodium/taurocholate co-transporting polypeptide [16,40,41]. Using radiolabelled cholic acid and taurocholic acid, we have determined that the amount of cholic acid that becomes associated with McNtcp cells is approx. 60% lower than that of taurocholic acid after overnight incubation in medium containing 100 μM of the bile acids (S. K. Cheema, M. Blacker-Forbes and L. B. Agellon, unpublished results). In these cells, only conjugated bile acids decreased the expression of the transgenes containing the sequence of the 3′-UTR of the mouse cyp7 mRNA and the effect was evident even at a concentration one-quarter of that of unconjugated bile acids. Unconjugated bile acids might not participate in the post-transcriptional regulation of the expression of the cyp7 gene.

The abundance of the rat cyp7 mRNA in rat hepatoma L35 cells is apparently not altered by physiological concentrations of bile acids, despite the ability of these cells to concentrate taurocholate from the medium [15]. However, supraphysiological concentrations (1 mM) of bile acids did decrease cyp7 mRNA abundance in L35 cells and the effect could be mimicked by SDS (50 μM). The decrease in cyp7 mRNA abundance was ascribed to cytotoxic effects on the basis of the activity of cytosolic enzymes in the culture medium. Repression of the CMV.CAT.CYP7 transgene expression in McNtcp cells was evident at concentrations of TCDCA in the medium that were in the lower range of physiological concentration and below the critical micellar concentration. The L35 cells might not have the correct complement of cellular factors to permit a response to physiological levels of bile acids in the culture medium.

The AREs, which are probably responsible for the short half-life of the cyp7 mRNA *in vivo*, seem to be distinct from the putative elements that are responsive to bile acids. Regulation at the post-transcriptional level provides an additional dimension in controlling the expression of the cyp7 gene. This mode of regulation also allows different species of bile acids to either enhance or minimize their inhibitory effect on the expression of the cyp7 gene.

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