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Regulation of Rabbit Cytochrome P450 2E1 Expression in HepG2 Cells by Insulin and Thyroid Hormone

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ABSTRACT

The regulation of cytochrome P450 (CYP) 2E1, the ethanol-inducible isoform, is particularly complex. The level is affected by a variety of other foreign compounds, by insulin (as studied in several laboratories), and by triiodothyronine (T_3), which has not been previously examined at the molecular level. In the present investigation, a stably transfected HepG2 cell line harboring a rabbit *CYP2E1* minigene containing the coding sequence together with 1.6 kilobases of the 5′ flanking region and the untranslated region (UTR), as well as 0.5 kilobases of the 3′ UTR, was established. Western blot analysis showed that 1 μM insulin decreased the CYP2E1 protein level in a dose- and time-dependent manner, whereas 1 μM T_3 increased the level 2-fold in 1 day and 8-fold in 5 days. Similarly, steady state CYP2E1 mRNA levels were decreased by insulin but were increased by T_3. Neither hormone affected the transcription rate of the *CYP2E1* 5′ flanking region with an UTR/luciferase fusion gene, indicating that the regulation is post-transcriptional in this system under our experimental conditions. When the *CYP2E1* 3′ UTR was removed from the minigene, CYP2E1 mRNA and protein were up-regulated by insulin but were not affected by T_3. These findings, including mRNA half-life determinations, indicate that the effects of insulin and T_3 are a result of altered mRNA stability and that the 3′ UTR of *CYP2E1* contains regulatory information for these hormone-mediated effects.

CYP2E1, which was first characterized as a unique, ethanol-inducible isoform purified from rabbit hepatic microsomes (Koop *et al.*, 1982), is now known to occur in a variety of tissues and species, including humans. The substrates metabolized by this particular oxygenase include the drug acetaminophen and numerous other foreign compounds, such as benzene, nitrosamines, and carbon tetrachloride, which yield toxic or carcinogenic products. Accordingly, the extent of induction of this isoform by ethanol and other chemical agents and its physiological regulation by hormones are of much interest. Early studies in several laboratories indicated that chemically elicited diabetes alters the metabolism of various xenobiotics in microsomes. It was later established that CYP2E1 is induced in diabetic rats (Song *et al.*, 1987; Bellward *et al.*, 1988; Dong *et al.*, 1988) and that the increases in both CYP2E1 mRNA levels and enzyme content are reversed by insulin administration (Dong *et al.*, 1988). However, there have been conflicting reports regarding the hormonal modulation of CYP2E1 levels in humans. Song *et al.* (1990) observed that CYP2E1 protein levels were elevated in lymphocytes from patients with insulin-dependent diabetes mellitus, but CYP2E1 activity measured as chlorzoxazone hydroxylation *in vivo* was not enhanced in patients with diabetes, compared with controls (Berthou *et al.*, 1997).

It is not clear which factors contribute predominantly to pathophysiological changes in CYP2E1 in the diabetic state. In addition to a decrease in insulin levels, several hormonal and metabolic consequences of diabetes, such as decreased growth hormone, glucagon, androgen, and thyroid hormone levels and increased serum glucose and ketone body levels, may also mediate the effect. At first, induction of CYP2E1 in diabetes was attributed to diabetic ketosis, directly or indirectly (Bellward *et al.*, 1988), but the degree of ketosis was insufficient to account for the observed effect. On the other hand, and in contrast to the finding of Yamazoe *et al.* (1989b), growth hormone administration to male rats failed to reverse the effect of diabetes (Thummel and Schenkman, 1990). These results suggest that more than one mechanism may be important in the induction of CYP2E1 in diabetes. Although daily treatment with insulin resulted in a decrease in CYP2E1 in liver microsomes from diabetic animals (Dong *et al.*, 1988), Johansson *et al.* (1991) found that the addition of insulin to primary rat hepatocytes caused a stabilization of the CYP2E1 protein, and similar results have been found in COS-7 cells (Freeman and Wolf, 1994). Subsequently, de

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ABBREVIATIONS: IRS, insulin response sequence; UTR, untranslated region; T_3, triiodothyronine; T_4, thyroxine; TRE, thyroid response element; MEM, minimal essential medium; FBS, fetal bovine serum; ECL, enhanced chemiluminescence; kb, kilobase(s); bp, base pair(s); RT, reverse transcription; PCR, polymerase chain reaction; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole.

Waziers *et al.* (1995) concluded that insulin directly down-regulates the expression of CYP2E1 in rat hepatoma cells. Woodcroft and Novak (1997) also demonstrated that decreases in the concentration of insulin in the culture medium resulted in increased CYP2E1 levels in primary rat hepatocytes.

The mechanism of CYP2E1 induction in diabetes has been shown to be pretranslational. In contrast to the post-translational effects of inducing agents of low molecular weight, such as ethanol and acetone, CYP2E1 mRNA levels were markedly increased in diabetic rats (Song *et al.*, 1987; Dong *et al.*, 1988). The former investigators further showed that the increase in mRNA levels was the result not of enhanced rates of transcription but of stabilization of the CYP2E1 mRNA; however, direct half-life measurements were not made. Weak repression of the transcription of CYP2E1 occurred when rat Fao cells were treated with insulin, although the major effect of insulin is acceleration of mRNA turnover (de Waziers *et al.*, 1995).

Most studies on the regulation of *CYP2E1* have been carried out with rats, but species differences in the responses of particular genes to pathophysiological regulation must be considered. In rabbits, the possible roles of insulin and thyroid hormone in the regulation of hepatic CYP2E1 levels have not been established. A putative IRS (TGGTTTTTGT) has been identified in rabbit *CYP2E1* (Khani *et al.*, 1988; Pernecky *et al.*, 1994); it is located between the TATAA box and the transcription start site, with three nucleotide mismatches, relative to the IRS for the phosphoenolpyruvate carboxykinase gene (TGGTGTTTTG). Two homologous TRE half-sites (AGGTCA) are also present in the 32-bp direct repeat in the 5′-flanking region of *CYP2E1*, with one mismatch, relative to the consensus TRE hexamer (GGGTCA). The presence of such sequences in the promoter does not indicate that these elements are functional. Therefore, the possibility of transcriptional control of the rabbit *CYP2E1* gene by insulin and thyroid hormone was studied by determination of the promoter activity after transient transfection.

The aim of the present investigation was to examine the effects of insulin and thyroid hormone on rabbit *CYP2E1* expression and to elucidate the molecular results of the possible regulation. A cell line stably expressing rabbit *CYP2E1*, under the control of its own promoter and 5′ flanking region, was established. Because a rabbit liver cell line was not available, we chose a human HepG2 cell line with very low endogenous CYP2E1 content. The results show that insulin down-regulates and thyroid hormone up-regulates rabbit CYP2E1 expression in HepG2 cells by affecting the mRNA half-life. The sequence in the 3′ UTR of *CYP2E1* contains the necessary information to mediate these effects.

Experimental Procedures

Materials. HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD). Human insulin and T_3 (both of tissue culture grade) were obtained from Sigma Chemical (St. Louis, MO), and pcDNA3.1 was from Invitrogen (Carlsbad, CA). MEM-α, FBS, transfection reagents, restriction enzymes, Klenow enzyme, Geneticin, and Superscript II were from Gibco BRL (Gaithersburg, MD). ECL detection systems for Western and Southern blots were from Amersham (Arlington Heights, IL), peroxidase-conjugated anti-sheep IgG was from Calbiochem (La Jolla, CA), and pBluescript II SK(+) was from Stratagene (La Jolla, CA). Plasmid and total RNA isolation kits were from Qiagen (Santa Clarita, CA), pGL3-basic, pRL-TK, and dual-luciferase reporter assay systems from Promega (Madison, WI), and *Thermus aquaticus* DNA polymerase and the thermal cycler from Perkin Elmer (Emeryville, CA). The imaging densitometer was from Bio-Rad (Hercules, CA).

Construction of rabbit *CYP2E1* minigenes. Full-length rabbit CYP2E1 cDNA was released by treatment of pSP3a with *Pvu*II (partial digestion) and *Xho*I, ligated to 1.58 kb of 5′ flanking region and UTR derived from the *Apa*I/*Pvu*II-digested *CYP2E1* genomic clone, subcloned into *Apa*I/*Xho*I sites of pBluescript II SK(+), and designated pAX. The *CYP2E1* 3′ UTR was obtained by PCR amplification with pSP3a as a template. The 5′ primer (AAGCAAGAATTCCCTGATCCCGAG) is located in positions 1135–1158 of rabbit *CYP2E1* (GenBank accession number M15061) and contains an *Eco*RI site, and the 3′ primer (GGGCCCTCTAGAGGGCTTGAAAGGTTACTGTTTATT) is located just before the poly(A)$^+$ tail and contains an *Xba*I adapter. pcDNA3.1 was modified by digestion with *Nru*I and *Hin*dIII to remove the cytomegalovirus promoter. Two rabbit *CYP2E1* minigenes were constructed; plasmid p2E1-A, which contains a 5′ flanking region with an UTR and full-length cDNA, was constructed by ligation of *Kpn*I/*Xho*I-digested pAX with promoterless pcDNA 3.1, and plasmid p2E1-B, which contains the 5′ flanking region with an UTR, full-length cDNA, and a 3′ UTR, was constructed by ligation of *Kpn*I/*Eco*RI-digested p2E1-A and *Eco*RI/*Xba*I-digested PCR products described above. The structures of all constructs were confirmed by sequencing. HepG2 cells were stably transfected with p2E1-A or p2E1-B in the presence of lipofectin, and Geneticin-resistant clones were selected.

Cell cultures. Stably transfected HepG2 clones were grown in MEM-α supplemented with 10% FBS and 0.25 mg/ml Geneticin. Cells were grown in 5% CO_2 at 37° to >90% confluency (seeded at 1×10^5 cells/cm^2), and serum was withdrawn for 24 hr before hormone treatment. A stock aqueous solution of insulin (1 mM) was prepared and stored at −20° until use. T_3 was dissolved in 1 N NaOH; the amount added (0.1% of the volume of the medium) did not affect the pH. The cells were refed with fresh MEM-α and T_3 each day when the effects of this hormone were studied.

Western blotting. Microsomes were obtained by differential centrifugation of sonicated cell lysates, and the protein contents were determined by the bicinchoninic acid method (Wiechelman *et al.*, 1988). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out with a Bio-Rad Mini PROTEAN II apparatus. Samples containing 10 μg of protein were blotted onto Hybond-ECL membranes and incubated with polyclonal antiserum to CYP2E1 and with peroxidase-conjugated anti-sheep IgG, as the primary and secondary antibodies, respectively. The ECL method was used for visualization of protein, and the intensities of the bands were quantified with a Bio-Rad imaging densitometer (model GS-670).

RT-PCR method and Southern blotting. Total RNA was isolated by RNeasy. First-strand cDNAs were synthesized with random hexamers and Superscript II, and 2 μl of the 10-fold diluted cDNA product was included in the PCR mixtures. A *CYP2E1* 5′ primer (CATCGGGAATCTTCTCCAGTTGG) corresponding to positions 60–82 and a *CYP2E1* 3′ primer (TGAAGGGTGTGCAGCCGATGACAA) complementary to bases 446–469 were used for amplification of 410 bp of the CYP2E1 product. The reaction mixtures were heated at 94° for 4 min and immediately cycled 32 times through a 1-min denaturing step at 94°, a 1-min annealing step at 55°, and a 2-min extension step at 72°. Human calmodulin primers or PCR-mimic served as an internal control. RT-PCR products were separated on 1.3% agarose gels and subjected to Southern blotting with a probe corresponding to nucleotides 80–222 of *CYP2E1*. Hybridization and detection were performed with the ECL direct nucleic acid labeling and detection kit. The intensities of the bands were quantified as described above for Western blots.

PCR-mimic construction of an internal standard. To construct an internal standard that contained the same primer template

sequences as the target, but differing in size, CYP2E1 cDNA clone pSP3a was digested with *Bam*HI/*Xba*I and separately digested with *Pvu*II/*Asp*700. The *Bam*HI/*Xba*I digest (3.4 kb) and the *Pvu*II/*Asp*700 digest (200 bp) were filled in with Klenow enzyme before ligation, resulting in a fragment 150 bp shorter than the original construct. PCR was performed as described, except that, in each tube, 1 μl of a known amount (0.05 amol) of the PCR-mimic competitor was added along with unknown amounts of cDNA. After PCR and Southern blotting, the products generated by the standard (260 bp) and target (410 bp) were quantified.

Transient transfection and reporter assay. HepG2 cells were cultured to >90% confluency in MEM-α supplemented with 10% FBS and were transiently transfected with 1.58 kb of *CYP2E1* 5′ flanking region and UTR (nucleotides −1576 to +33) linked to the pGL3-basic luciferase reporter vector, using lipofectamine. Five hours after the addition of DNA, cells were refed with medium containing 10% dialyzed FBS and the indicated amounts of hormones, for 42 hr. A dual-luciferase reporter assay system was used to detect promoter activity, with *Renilla* luciferase (pRL-TK) as an internal standard for determination of transfection efficiency.

mRNA half-life determination. To transfected HepG2 cells treated with a hormone or left untreated for 12 hr, the transcription inhibitor DRB was added to a final concentration of 100 μM. Total RNA was isolated at different times (0–24 hr) after DRB treatment, and RT-PCR followed by Southern blotting were carried out as described above. The amount of CYP2E1 mRNA at time 0 in each group was assigned a value of 100, and all other results were expressed as percentages of this value. Half-life values were calculated from first-order decay rate constants.

Results

To examine the regulation of rabbit *CYP2E1* expression, a minigene (p2E1-B) was constructed that contained the coding region of the full-length rabbit CYP2E1 cDNA, 1.58 kb of the 5′ flanking region with an UTR, and 480 bp of the 3′ UTR; no intron was included in this construct (Fig. 1). HepG2 cells were stably transfected with p2E1-B; after selection for Geneticin resistance, two clones that exhibited the highest CYP2E1 protein expression were isolated and designated B4 and B8. The expression levels were 0.2 pmol of CYP2E1/mg of microsomal protein for clone B4 and 1 pmol of CYP2E1/mg of microsomal protein for clone B8. The two clones responded similarly to physiological agents, but B4 showed greater induction upon treatment with T_3 and was therefore investigated further. Stably transfected HepG2 cells are preferable to transiently transfected cells because the genes are integrated into chromosomes whose structures are known to influence gene transcription and regulation.

The effects of hormones on CYP2E1 content are depicted in Fig. 2A. HepG2 cells without the insert showed no detectable CYP2E1 signal, whereas clone B4 contained a protein with molecular mass (51 kDa) identical to that of highly purified CYP2E1 from rabbit liver microsomes. The amount of CYP2E1 decreased upon addition of insulin but showed a substantial increase when T_3 was added to the medium; the T_3-mediated increase was attenuated when insulin was included together with T_3. The results of densitometric quantification of the bands in eight such experiments are given in Fig. 2B. Insulin and T_3 produced an approximately 50% decrease and an approximately 4-fold increase in the CYP2E1 levels, respectively, and the enhancement by T_3 was halved by insulin. The down-regulation of CYP2E1 by insulin displayed a time dependence, with a >50% decrease being seen after 3 days of cell treatment (Fig. 3A); maximal repression of CYP2E1 was observed with 10^{-6} M insulin (Fig. 3B). Time course experiments with T_3 revealed an initial lag phase and a subsequent gradual increase in the amount of CYP2E1, which was almost 8-fold after 5 days of exposure to the hormone (Fig. 4A). Furthermore, CYP2E1 synthesis was increased 2.5-fold with concentrations of T_3 as low as 10^{-9} M, the response was greater at higher hormone concentrations, and saturation occurred with approximately 10^{-6} M T_3 (Fig. 4B). The expression of CYP2E1 was also stimulated by T_4, but only 60% as effectively as by T_3 after 3 days of cell treatment; in general, approximately 10 times more T_4 than T_3 was required for maximal stimulation of *CYP2E1* expression (Fig. 4B).

To evaluate hormonal effects on *CYP2E1* expression at the transcript level, RT-PCR and Southern blotting were performed, because Northern blots are not sufficiently sensitive for detection of CYP2E1 mRNA changes in this system. As illustrated in Fig. 5, insulin caused down-regulation of CYP2E1 mRNA beginning at 6 hr of incubation. In contrast, treatment with T_3 for the same period resulted in a marked

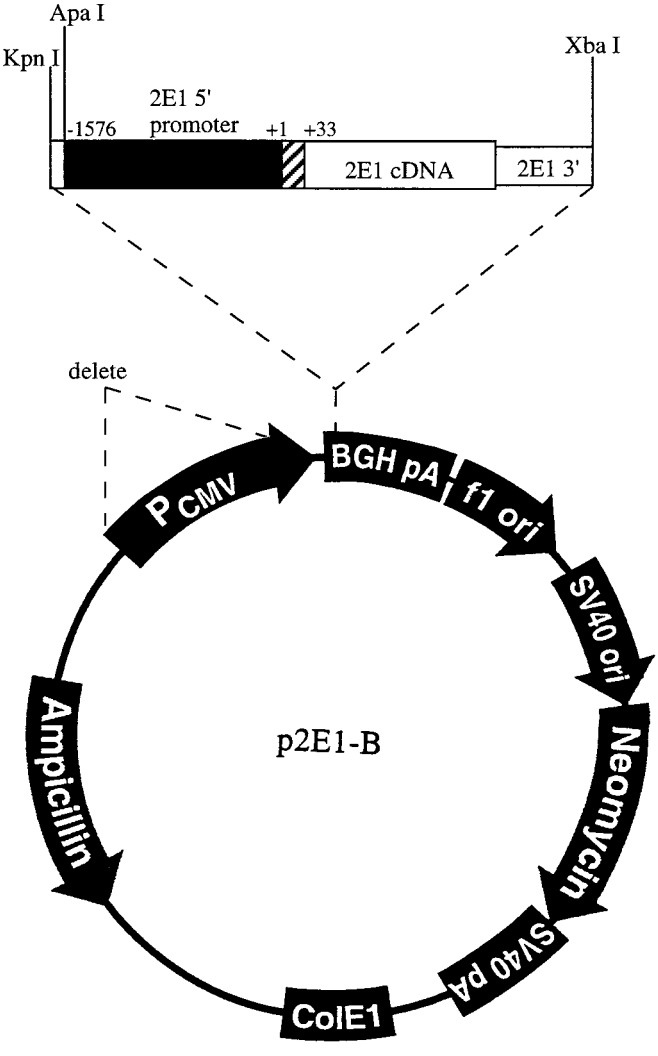

Fig. 1. Restriction map and construction strategy for preparation of the rabbit *CYP2E1* minigene. The construct (p2E1-B) contains the 5′ flanking region with UTR sequences from nucleotide −1576 to nucleotide +33, the full coding region, and 480 bp of the 3′ UTR. *CMV*, cytomegalovirus; *BGH*, bovine growth hormone; *pA*, polyadenylation signal; *ori*, origin; *SV40*, simian virus 40; *ColE1*, *E. coli* origin of replication.

increase in the amount of CYP2E1 message. The finding that CYP2E1 mRNA and protein levels responded similarly to insulin and T_3 suggests that the regulation of CYP2E1 by these hormones is pretranslational. The steady state level of mRNA represents the balance of synthesis, nuclear processing, transport from the nucleus to the cytoplasm, and cytoplasmic degradation. To investigate whether transcriptional regulation is involved, a 1.58-kb fragment of the 5′ flanking region and UTR of rabbit *CYP2E1* was fused to the luciferase reporter gene (Fig. 6A), and the chimeric construct was transiently transfected into HepG2 cells and assayed for promoter activity. Neither insulin nor T_3 affected this activity (Fig. 6B), indicating that the regulation is post-transcriptional in this system under our experimental conditions.

Because the regulation of CYP2E1 expression by insulin and T_3 is apparently unrelated to transcription, the possibility of mRNA stabilization was considered. Taking into account the well-known role of the 3′ UTR in transcript stabilization (Jackson, 1993), a rabbit *CYP2E1* minigene derivative that lacked the *CYP2E1* 3′ UTR was constructed. Construct p2E1-A, containing the same 1.58-kb upstream and coding region segments as clone B4 but no *CYP2E1* 3′ UTR, was stably transfected into HepG2 cells, and a clone (A9) that expressed 0.2 pmol of CYP2E1/mg of microsomal protein was selected. Deletion of the 3′ UTR from the *CYP2E1* minigene (clone A9) profoundly altered the hormonal response. In this connection, insulin appreciably increased the amount of CYP2E1 protein formed, whereas the stimulation previously observed with T_3 was abolished with clone A9, which lacks the *CYP2E1* 3′ UTR (Fig. 7). Also with clone A9, transcript levels mirrored the sharp increase in CYP2E1 protein levels seen with insulin treatment, and T_3 was without effect (Fig. 8).

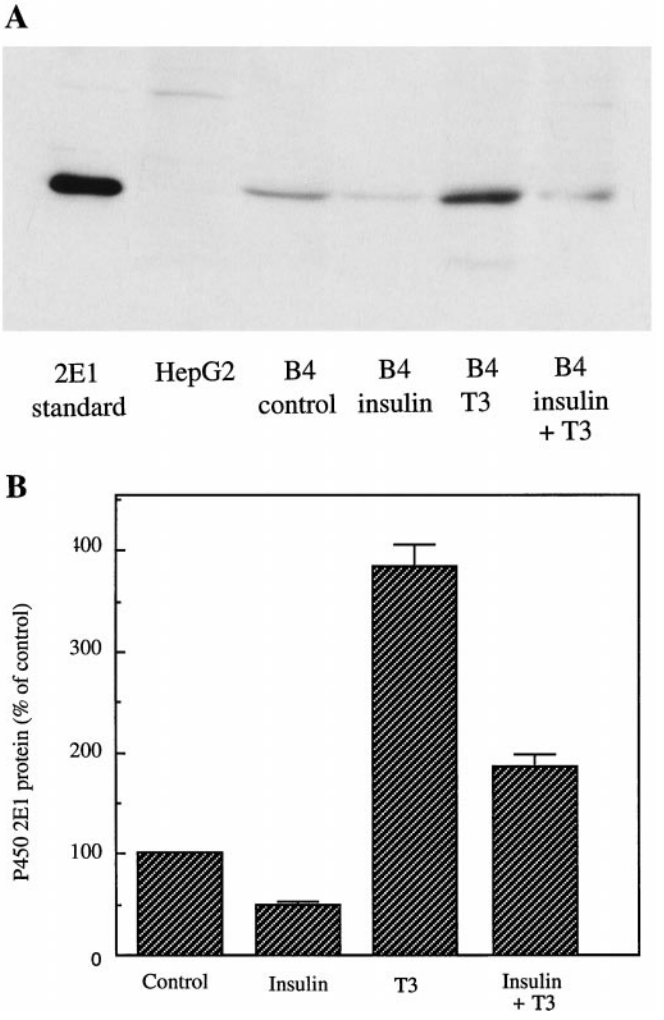

Fig. 2. A, Western blot determination of CYP2E1 in microsomes obtained from the B4 clone (with the *CYP2E1* 3′ UTR), after culture with added hormones, as indicated, for 3 days. When present, insulin (1 μM) was added on the first day and T_3 (1 μM) on each day. The amounts of protein were 1 ng for purified CYP2E1 standard and 10 μg for microsomal preparations. B, Effects of insulin and T3 treatment on the amounts of CYP2E1 protein expressed by the B4 clone. The results are the mean ± standard error of eight separate experiments.

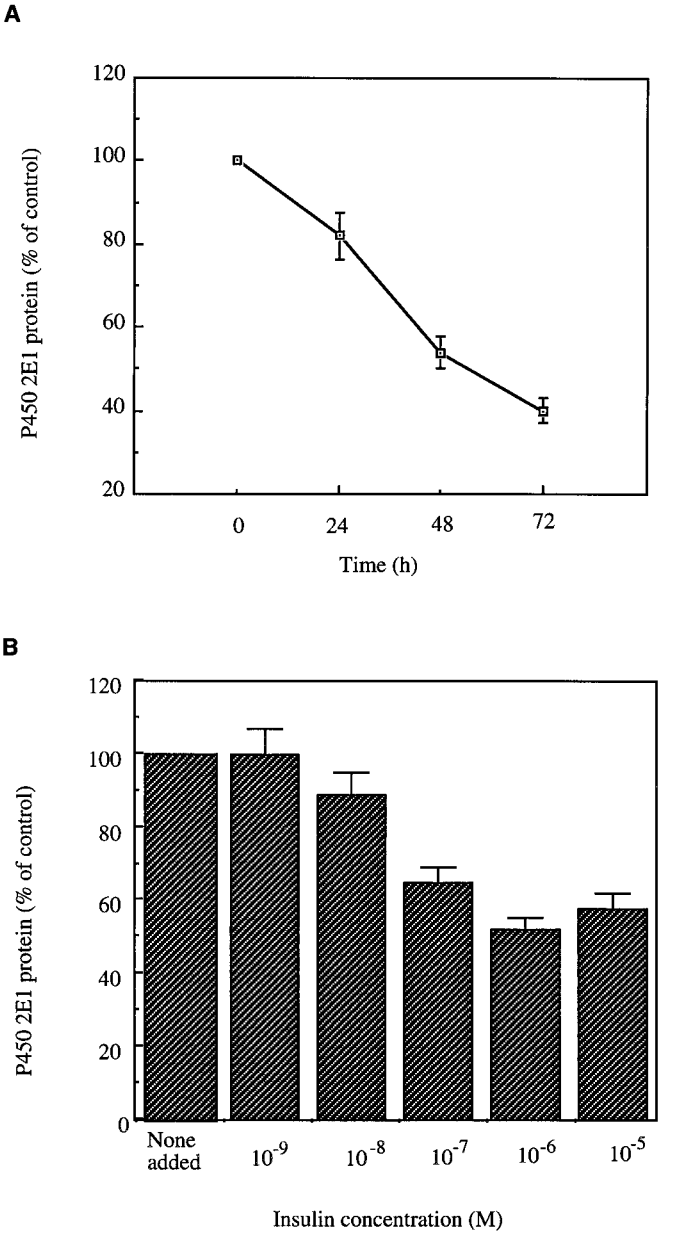

Fig. 3. A, Time course of the effects of 1 μM insulin on the amount of CYP2E1 protein expressed by the B4 clone. B, Concentration-dependent effects of insulin on the amount of CYP2E1 protein expressed at the end of 3 days. The results are the mean ± standard error of three separate experiments.

To ascertain whether the hormone-elicited changes in CYP2E1 mRNA steady state levels resulted from differences in the rates of degradation, transcript half-life determinations were made with cells incubated with DRB to arrest transcription (actinomycin D was not used because of its toxicity to the cells at the desired concentration). With clone B4, which contains the *CYP2E1* 3′ UTR, insulin down-regulated the expression of CYP2E1 by shortening the mRNA half-life from 9.6 ± 0.3 to 5.4 ± 0.3 hr, whereas T_3 up-regulated CYP2E1 by lengthening message half-life to 20.1 ± 2.5 hr (Fig. 9). The mRNA half-life of 9.6 ± 0.3 hr obtained with clone B4 (with no additions) (Fig. 9) was decreased to

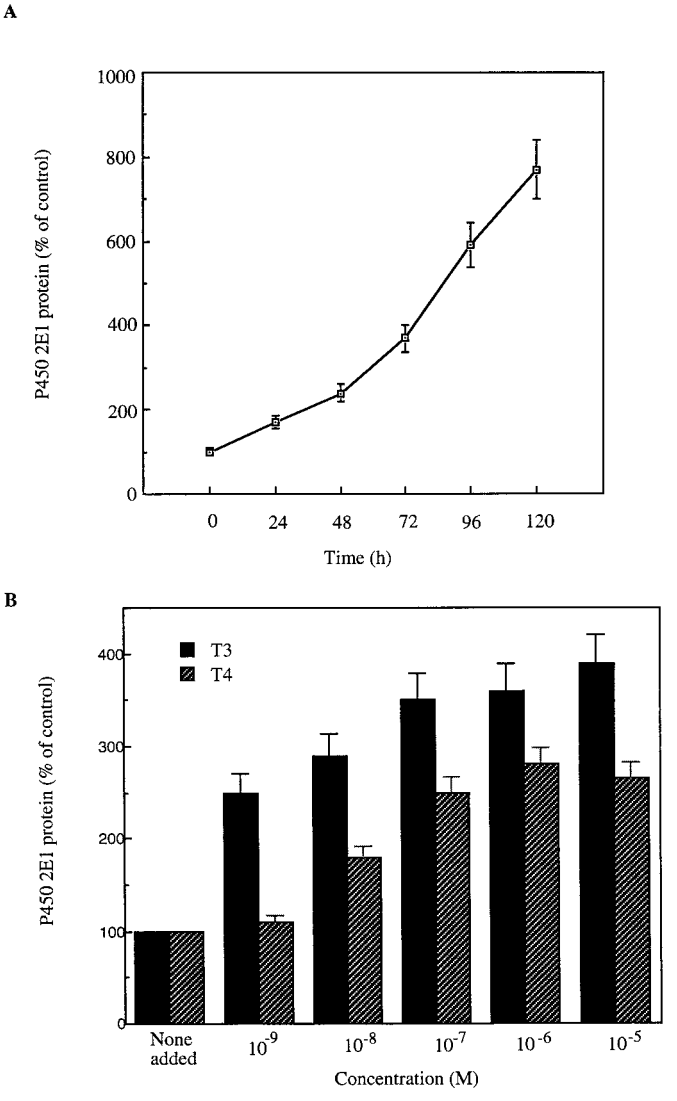

Fig. 4. A, Time course of the effects of 1 μM T_3 on the amount of CYP2E1 protein expressed by the B4 clone. B, Concentration-dependent effects of T_3 and T_4 on the amount of CYP2E1 protein expressed at the end of 3 days, with daily replacement. The results are the mean ± standard error of three separate experiments.

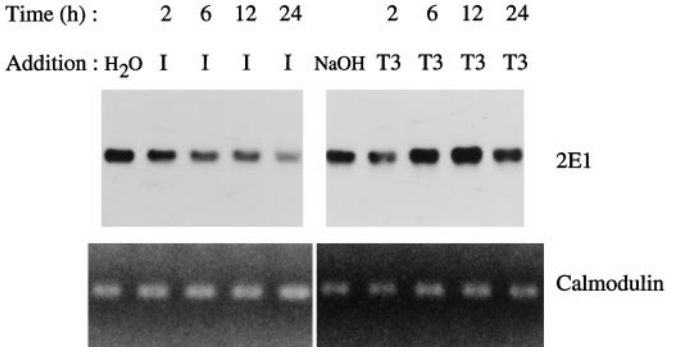

Fig. 5. RT-PCR followed by Southern blot analysis of total RNA (2.5 μg) prepared from the B4 clone. Treated cells were exposed to 1 μM insulin (*I*) or 1 μM T_3 for the times indicated. Human calmodulin was amplified from the same diluted cDNA, to serve as an internal standard for evaluation of the results with CYP2E1.

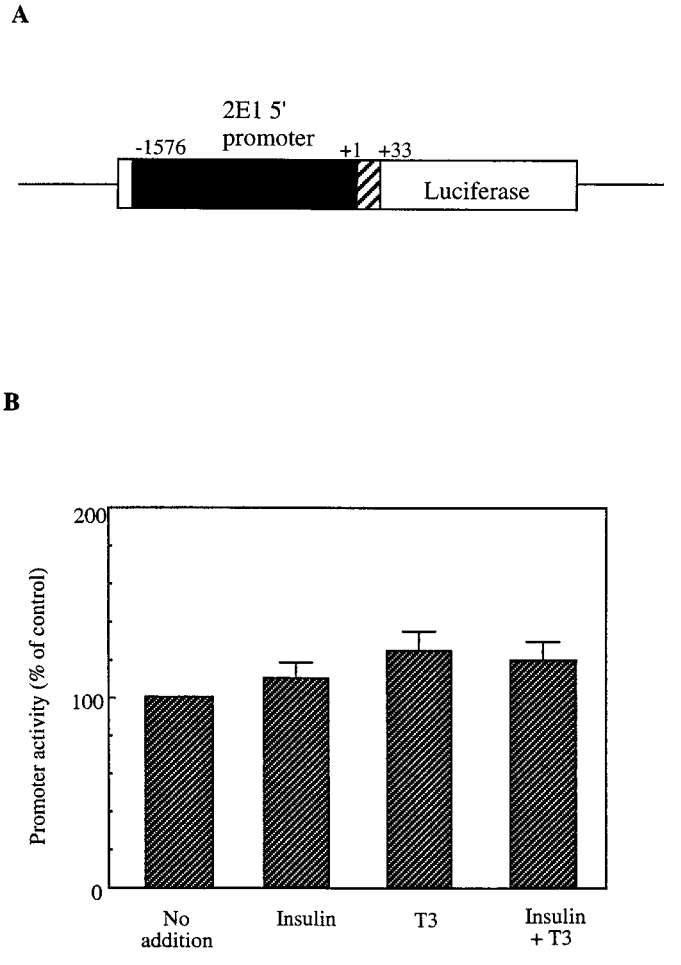

Fig. 6. A, Rabbit *CYP2E1* promoter (nucleotides −1576 to +33)/luciferase chimeric gene construct. B, Luciferase activity of HepG2 cells transiently transfected with the *CYP2E1* promoter/luciferase chimeric gene, in the absence or presence of 1 μM insulin or T_3, for 42 hr. Normalized promoter activities after such treatment are expressed as percentages of the luciferase activity in untreated HepG2 cells. The results represent the mean ± standard error of three independent determinations.

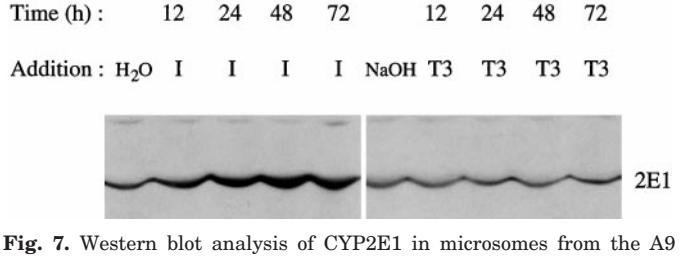

Fig. 7. Western blot analysis of CYP2E1 in microsomes from the A9 clone (without the *CYP2E1* 3′ UTR), cultured in the presence of 1 μM insulin (*I*) or T_3 for up to 72 hr.

6.1 ± 0.8 hr with clone A9, which lacks the *CYP2E1* 3′ UTR (with no additions) (Fig. 10). Inclusion of insulin with the A9 clone resulted in almost doubling of the half-life (to 10.0 ± 1.1 hr), and T_3 apparently had no significant effect (6.9 ± 0.7 hr) (Fig. 10). In other experiments not shown, degradation half-lives for the CYP2E1 protein were not appreciably affected by insulin or T_3. These observations suggest that alterations in CYP2E1 mRNA stability may account for the modulation of CYP2E1 by insulin and T_3 and, furthermore, that the 3′ UTR of *CYP2E1* contains structural information governing transcript stability.

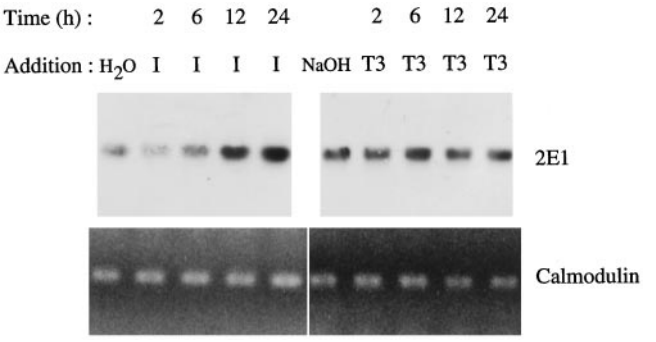

Fig. 8. RT-PCR followed by Southern blot analysis of total RNA (2.5 μg) prepared from the A9 clone; treated cells received 1 μM insulin (*I*) or 1 μM T_3 from 2 to 24 hr. Human calmodulin was amplified from the same diluted cDNA, to serve as an internal standard.

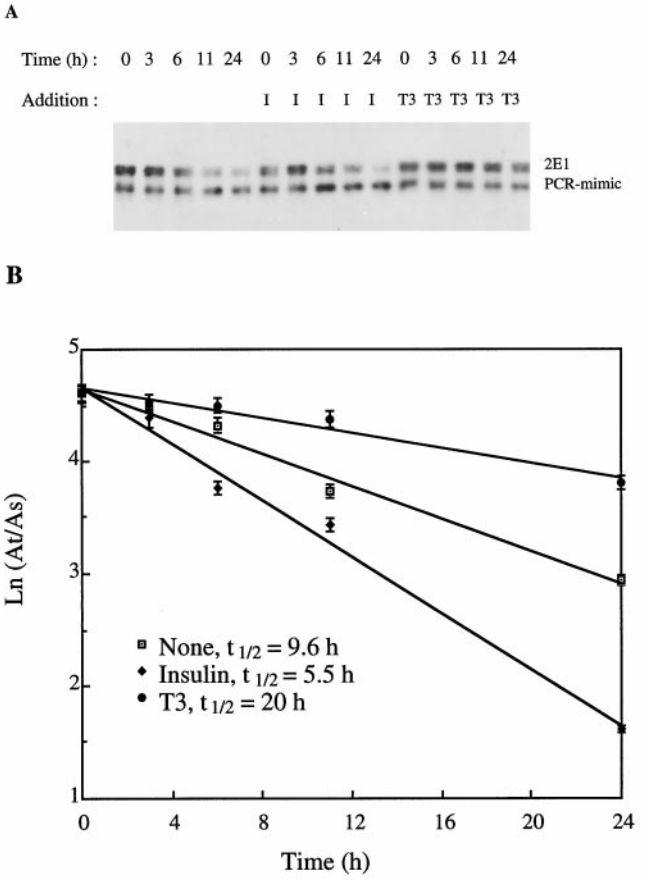

Fig. 9. A, Decay of CYP2E1 mRNA in the B4 clone, as analyzed by quantitative RT-PCR followed by Southern blotting. Total RNA was isolated from the B4 clone at the indicated times after addition of DRB, with or without hormone treatment. A constant amount of competitor (PCR-mimic) was coamplified with the target cDNA. The 410-bp amplification product was generated from CYP2E1 RNA, and the 260-bp product was generated from the CYP2E1 PCR-mimic, as an internal control. *I*, Insulin. B, Decay of CYP2E1 mRNA with time in the presence of DRB. Three separate experiments were carried out, and a representative semilogarithmic plot of the percentage of the time 0 values versus time is shown. The best fit line was determined by linear regression analysis, and half-lives were calculated from first-order decay rate constant ($t_{1/2}$ = 0.693/k). *At*, amount of target (ZE1); *As*, amount of standard (PCR-mimic).

Discussion

The regulation of *CYP2E1* is a very complex process. The major process that provides control in adult animals is post-transcriptional (Koop and Tierney, 1990), in contrast to the transcriptional activation that begins shortly after birth and reaches a maximum a few days later (Umeno *et al.*, 1988b). In the present study, changes in the CYP2E1 mRNA levels in response to hormonal manipulations paralleled the changes in the CYP2E1 protein levels, indicating that the hormonal control occurs at the pretranslational level. Although weak transcriptional repression by insulin was reported in rat hepatoma cells (de Waziers *et al.*, 1995) and an homologous IRS is found in the rabbit *CYP2E1* gene sequence (nucleotides −17 to −8) (Khani *et al.*, 1988), as well as in human *CYP2E1* (nucleotides −2634 to −2625) (Umeno *et al.*, 1988a) and in rat *CYP2E1* (nucleotides −1434 to −1425) (Umeno *et*

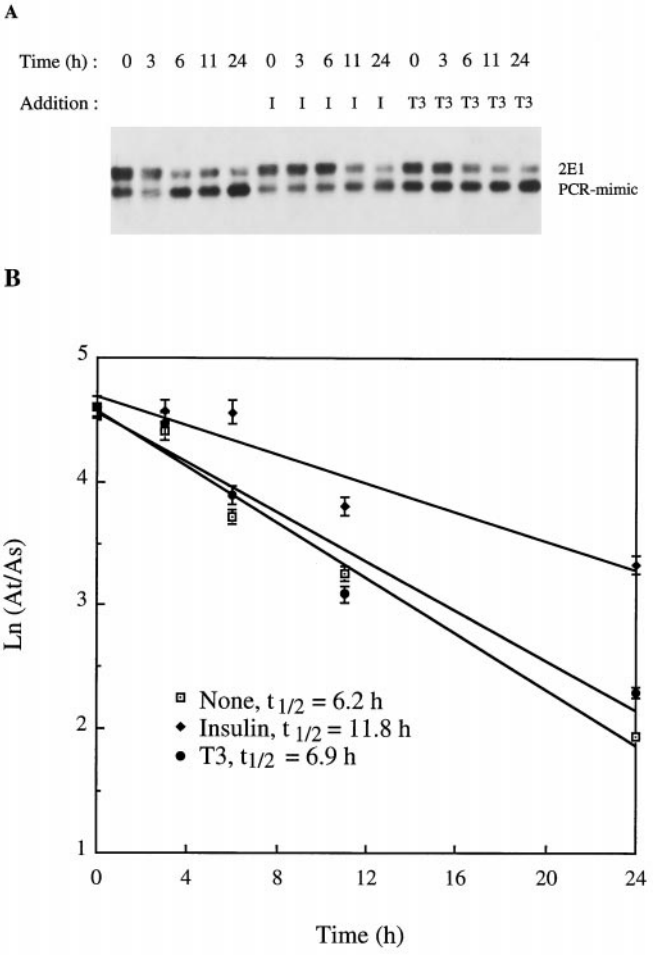

Fig. 10. Decay of CYP2E1 mRNA in the A9 clone, as analyzed by quantitative RT-PCR followed by Southern blotting. The experimental conditions and analysis were as described in the legend to Fig. 9.

al., 1988b), the involvement of such a sequence in the regulation of rabbit *CYP2E1* by insulin is not supported by the reporter gene activities presented here. On the other hand, mRNA destabilization may be the most important factor in the regulation of CYP2E1 in response to insulin.

Thyroid hormone at high doses has been shown to suppress the levels of total hepatic cytochrome P450 (Skett and Weir, 1983), and smaller amounts selectively suppress the levels of some individual isoforms (Yamazoe *et al.*, 1989a). It has also been reported that rat CYP2E1 levels are elevated 3–5-fold after hypophysectomy (Waxman *et al.*, 1989) and that treatment with T_3 results in marked depletion of CYP2E1 in the liver of thyroidectomized rats (Rosenberg *et al.*, 1995). However, the direct effect of T_3 has not been studied. In this study, we have found that T_3 (or T_4) dramatically increases CYP2E1 mRNA and protein contents in stably transfected HepG2 cells and that it is the 3′ UTR and not the putative TRE in the 5′ flanking region of rabbit *CYP2E1* that is involved in this regulation.

Insulin regulates mammalian genes in many ways, including effects on transcription initiation, mRNA stability, and protein half-life (O'Brien and Granner, 1991). This hormone has been shown to stabilize glycerol-3-phosphate dehydrogenase mRNA (Dani *et al.*, 1989) and pyruvate kinase mRNA (Decaux *et al.*, 1989), but in most other cases the step at which post-transcriptional regulation occurs is unknown. Studies of thyroid hormone-responsive genes have also demonstrated multiple levels of control (Glass and Holloway, 1990). Increased mRNA half-life in response to T_3 administration has been documented for rat growth hormone (Diamond and Goodman, 1985) and malic enzyme genes (Song *et al.*, 1988). Although it is well established that transcriptional control represents a direct action of thyroid hormones through the thyroid hormone receptor, in no case has the post-transcriptional action of thyroid hormones been demonstrated to directly involve the T_3 receptor, and the mechanisms responsible for these effects remain largely unknown (Glass and Holloway, 1990).

It is well accepted that mRNA destabilization is the major mechanism responsible for insulin regulation of CYP2E1 in rats. However, the involvement of the 3′ UTR in this regulation has never been examined directly. In the present study, the effects of both the 5′ flanking region with an UTR and the 3′ UTR in rabbit *CYP2E1* minigenes have been assessed, and the significance of the 3′ UTR in response to hormonal treatment has been demonstrated for the first time. The importance of this region in a key control mechanism has become more well recognized in the past decade. The 3′ UTR is involved in the regulation of mRNA in many ways, e.g., cellular localization, stabilization, and translational efficiency (Jackson, 1993). In the current investigation, deletion of the 3′ UTR of *CYP2E1* decreased mRNA stability, as reflected by mRNA half-life determinations. The degradation rate for A9 mRNA with the bovine growth hormone polyadenylation signal was 6.2 hr, which was faster than that for B4 mRNA containing the *CYP2E1* 3′ UTR (9.6 hr). Structural motifs in the 3′ UTR regulate mRNA stability through the AU-rich region or stem-loop secondary structures (Sachs, 1993), but no AUUUA mRNA destabilization motif was discernible in the rabbit *CYP2E1* 3′ UTR, and the way in which the stability of CYP2E1 mRNA is controlled by insulin and T_3 remains unknown.

Malter (1989) suggested that the rate of mRNA degradation is determined by the strength of the destabilizing sequences, rather than the stabilizing sequences. In some cases, sequence elements that silence destabilizing elements have been found, but in no case has a sequence, by itself, been found to confer stability on another mRNA (Sachs, 1993). Studies on the regulation of transferrin receptor mRNA decay (Klausner *et al.*, 1993) in response to changes in cellular stores of iron provide important mechanistic insight. Transferrin receptor mRNA is stabilized under the condition of iron starvation and is destabilized by iron. Located within the 3′ UTR of this mRNA is a region that contains five distinct stem-loop structures capable of binding the iron response element-binding protein. The affinity of this binding protein for the iron response element is regulated by cellular iron. It is currently believed that binding of the protein to the iron response element brings about stabilization of the transcript by protecting the mRNA from degradation. Transcript stabilization may, in fact, result from inhibition of the activity of a factor recognizing the destabilizing element in the 3′ UTR (Klausner *et al.*, 1993).

In a search for a sequence element in rabbit CYP2E1 mRNA that might modulate decay rates in response to hormones, a potential hairpin loop located several nucleotides before the polyadenylation signal was found in the rabbit 3′ UTR. However, experiments with progressive deletion constructs of the 3′ UTR are needed to demonstrate the involvement of such an element in hormonal regulation. Moreover, in a comparison of different species, it was found that the *CYP2E1* 3′ UTR is much longer in the rabbit sequence (480 bp) than in the human (152 bp) or rat (130 bp) sequences, and several common motifs are shared among different species in some regions. It is possible that 480 bp of the rabbit *CYP2E1* 3′ UTR contain a specific sequence or secondary stem-loop structure that interacts with a special protein that is induced by hormone treatment and modulates mRNA turnover. After a minimal sequence or structure in the 3′ UTR has been determined, a cytoplasmic protein that binds specifically to this sequence or structure could be isolated and characterized.

In summary, our study has addressed the post-transcriptional regulation by insulin and T_3 of *CYP2E1* expressed in HepG2 cells, especially the effects on mRNA turnover and the importance of the 3′ UTR in *CYP2E1* regulation. Additional experiments that delineate the function of the *CYP2E1* 3′ UTR will provide greater insight into the post-transcriptional regulation of *CYP2E1* by the 3′ UTR.

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