

Transcriptional Induction of Hepatic NADPH: Cytochrome P450 Oxidoreductase by Thyroid Hormone

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ABSTRACT

Studies were carried out to elucidate the mechanism whereby thyroid hormone (T3) induces NADPH:cytochrome P450 oxidoreductase (P450R) mRNA in rat liver *in vivo*. Northern blot analysis revealed that T3 treatment increases unspliced liver nuclear P450R RNA 4-fold within 8 h and that this induction precedes the induction of mature, cytoplasmic P450R RNA. Unspliced nuclear P450R RNA was suppressed below basal levels 24 h after T3 treatment, despite the continued presence of elevated circulating T3 levels. To determine whether the T3-stimulated increase in nuclear P450R RNA reflects an increase in P450R transcription initiation, nuclear run-on transcription assays were carried out. T3 induced a 6- to 8-fold increase in P450R transcription rate within 12 h, sufficient to account for the observed increase in nuclear P450R precursor RNA, followed by a decrease back to basal transcription levels

at 24 h, consistent with the nuclear RNA profile. Similar transcriptional increases were observed in nuclear run-on transcription studies using hybridization probes corresponding to nine different fragments of the P450R gene, spanning exon 2 to exon 16. Thus, P450R transcription initiation, not transcription elongation, is the T3-regulated event. Similar results were obtained during short (5 min) compared with long (45 min) nuclear run-on transcription assays, suggesting that changes in nuclear RNA processing or regulated degradation do not contribute to the overall RNA induction. This finding was confirmed by the ability of the RNA polymerase inhibitor actinomycin D, administered *in vivo*, to block T3 induction of P450R transcriptional activity. We conclude that P450R transcription, rather than nuclear RNA processing or mRNA stabilization, is the primary mechanism whereby T3 induces hepatic P450R mRNA.

NADPH:P450 reductase (P450R) is a microsomal flavoprotein that catalyzes the transfer of electrons from NADPH to microsomal cytochrome P450 (Porter, 1991; Strobel et al., 1995). P450R is encoded by a single gene containing 16 exons (Porter et al., 1990; O'Leary et al., 1994) that is transcribed from a TATA-less promoter (O'Leary et al., 1996) and is highly conserved in its domain structure across species (Sevrioukova and Peterson, 1995). P450R is expressed in liver and many extrahepatic tissues, where it provides electrons required for xenobiotic metabolism catalyzed by a large number of individual cytochromes P450. P450R is an obligatory and often rate-limiting component of microsomal P450-dependent activities, including drug metabolism and carcinogen bioactivation (Miwa et al., 1978; Kaminsky and Guengerich, 1985; Cawley et al., 1995). P450R also participates in P450-independent metabolic processes, including the metabolism and redox cycling of quinones, catechols, and related carcinogens (Chesis et al., 1984; Bligh et al., 1990; Sawamura et al., 1996). These processes are associated with the generation of reactive oxygen species and can contribute

to the cell's adaptive response to oxidative stress (Chen and Cederbaum, 1997; Piette et al., 1997).

Although the regulation of individual cytochrome P450 enzymes in response to exogenous and endogenous factors (e.g., drugs and other xenobiotics, developmental factors, and hormonal status) that may influence carcinogen activation and other P450 metabolic processes has been studied extensively at the molecular level (Waxman, 1999), much less is known about the regulation of P450R by endogenous hormones and other regulatory factors. One important endogenous regulator of P450 metabolism is thyroid hormone, T3, which not only contributes to the regulation of certain hepatic P450 enzymes (Ram and Waxman, 1990; Ram and Waxman, 1991) but is also a key determinant of the expression of P450R in liver and other tissues. Hepatic P450R activity, protein (Waxman et al., 1989), and mRNA (Ram and Waxman, 1992) are each decreased up to 5-fold after surgical removal of the pituitary (hypophysectomy) or by treatment of rats with the antithyroid drug methimazole (Ram and Waxman, 1992), and can be substantially restored by physiologic replacement of thyroxine (T4) but not other pituitary-dependent hormones. The down-regulation of P450R under conditions of thyroid hormone insufficiency is also observed in

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certain extrahepatic tissues, including lung (Ram and Waxman, 1992), a classic thyroid nonresponsive tissue (Dozin et al., 1985), and may be a part of the general decrease of basal metabolic activity seen in the hypothyroid state. Hypothyroidism is associated with a substantial decrease in cytosolic malic enzyme (Dozin et al., 1985; Lanni et al., 1995), which generates the NADPH necessary to support P450R-dependent metabolic activity, suggesting a metabolic basis for the apparent coordinate regulation of these two enzymes by thyroid hormone status. The decrease in hepatic P450R levels in hypothyroid rats also leads to substantial decreases in hepatic cytochrome P450-dependent steroid and xenobiotic metabolic activity as a consequence of the decrease in P450R enzymatic activity (Waxman et al., 1989). Studies using the antithyroid drug propylthiouracil confirm the thyroid hormone dependence of hepatic P450R and, additionally, make the important observation that the loss of P450R in hypothyroidism protects the liver from ethanol-induced liver injury associated with P450R-dependent redox cycling and oxidative stress (Ross et al., 1995). This latter finding may in part account for the striking effectiveness of anti-thyroid drugs in treatment of alcohol-induced liver disease (Orrego et al., 1994).

Previous investigations on the mechanisms by which thyroid hormone regulates P450R expression identified a functional thyroid response element in the P450R promoter and showed it to be composed of an imperfect repeat of the thyroid response motif AGGTCA (O'Leary et al., 1997). Other analyses revealed much larger increase in liver P450R mRNA in euthyroid rats given a thyroid receptor-saturating dose of T3 than could be accounted for in run-on transcriptional assay, suggesting that post-transcriptional regulatory mechanisms may also be involved (O'Leary et al., 1997). The present study was designed to further elucidate the regulatory actions of thyroid hormone on P450R expression at the level of P450R transcription initiation, transcription elongation, and nuclear RNA processing. Data are presented to support the conclusion that T3 stimulates, within 8 to 12 h, increases in P450R transcription initiation and corresponding increases in unspliced nuclear P450R RNA levels that precede the observed increase in cytoplasmic P450R mRNA. P450R transcription and nuclear RNA accumulation are further shown to subsequently be suppressed as a secondary response to hyperthyroidism, suggesting a mechanism to limit the induction of P450R and maintain hepatic P450R levels within physiologically acceptable limits.

Materials and Methods

Animals. Male Fischer rats were maintained under standard conditions of light and temperature. T3 was injected (200 μ g/100 g of BW, i.p.) and the rats killed 8 to 36 h later, as indicated. In some experiments, a second T3 injection was given 24 h after the first injection. In other experiments, actinomycin D (2.5 mg/kg BW; Sigma, St. Louis, MO) was coadministered with T3 to selectively inhibit RNA polymerase II (Connor et al., 1996). Plasma T3 levels were measured by radioimmunoassay using a commercial kit (Coat-A-Count canine T3 kit; Diagnostic Products Corp., Los Angeles, CA).

Nuclear RNA Isolation. Liver nuclei were isolated from T3-treated rats and from untreated or vehicle-injected rats and then stored frozen in liquid N₂ (Sundseth et al., 1992). Nuclear RNA was prepared from the frozen nuclei by a CsCl purification method (Kingston et al., 1996). Briefly, $\sim 10^7$ nuclei were homogenated in buffer D

(4 M guanidinium thiocyanate, 0.1 M TrisCl, pH 7.5, 1% 2-mercaptoethanol). Sodium lauryl sarcosinate [20% (w/v) in water] was added to the homogenate to a final concentration of 0.5%. The mixture was centrifuged at 5000g for 10 min at room temperature. The supernatant was layered onto a cushion of 5.7 M CsCl containing 0.01 M EDTA, pH 7.5, then centrifuged at 20°C at 100,000g. RNA pellets (yield ~ 20 μ g) were dissolved in DEPC-treated water and stored at -70°C until use.

Nuclear Run-on Analysis. Run-on transcription assays using nuclei isolated from untreated and from T3-treated rats were carried out using the general methods, including hybridization of washing conditions, described elsewhere (Tukey and Okino, 1991; O'Leary et al., 1997). Each sample contained 0.2 ml of liver nuclei (nuclei at a concentration of $A_{260} = 10$), 0.15 ml of nuclear run-on reaction buffer, 0.015 ml of nucleotide mix, and RNase inhibitor, creatine phosphate, creatine kinase, dithiothreitol, and 50 μ Ci of [α -³²P]UTP (3000 Ci/mmol) in a total volume of 0.4 ml (Tukey and Okino, 1991). Reactions were incubated for 30 min at 30°C unless indicated otherwise. Hybridization probes corresponding to different segments of P450R cDNA or the P450R gene were prepared by PCR using a RoboCycler 40 thermal cycler (Stratagene, La Jolla, CA). PCR reactions used P450R cDNA or either rat genomic DNA or reverse-transcribed, T3-induced rat liver nuclear RNA as template. Genomic DNA was prepared from rat liver by standard methods (Strauss, 1998). Oligonucleotide primers were selected to amplify specific exonic and intronic sequences of the P450R gene (Porter et al., 1990), as specified in Table 1. PCR was carried out as described (Beverley, 1992) with the following changes: 1) steps 7 to 11 were replaced by a single heating step (90°C for 3 s) and 2) in step 14, the conditions were changed to 2 min annealing at 55°C, 2 min extension at 72°C, and a denaturation time of 1.5 min. PCR products were separated from primers and templates by agarose gel electrophoresis. PCR products were excised from the gel, placed in dialysis tubing, eluted by gel electrophoresis, spin-dry concentrated, precipitated with isopropanol, and redissolved in water. Purified PCR products and linearized plasmid DNAs used in the nuclear run-on assay (1 μ g of PCR product or 5 μ g of plasmid DNA per filter slot) were denatured by adding NaOH to 0.1 M, followed by incubation at 25°C for 30 min. DNA samples were then slotted onto a GeneScreen nitrocellulose membrane using a Minifold II slot blot apparatus (Schleicher and Schuell, Keene, NH). The membrane was exposed to UV light for 1 min using a UV Stratalinker 240 instrument (Stratagene, Inc., La Jolla, CA) and stored at -20°C until use.

Labeled RNA transcripts generated in the nuclear run-on reaction were hybridized to GeneScreen membrane filters followed by a series of washings (O'Leary et al., 1997), with the following modifications. Prehybridization was at 65°C for 1 h, followed by hybridization at 65°C with 2 to 6 $\times 10^6$ cpm/filter for 36 h. The filters were washed in high-salt buffer (O'Leary et al., 1997) except that the washing was performed at 65°C for 1 h followed by a second wash at 37°C for 30 min with RNase solution (15 ml of 1 μ g/ml RNase A in 10 mM TrisCl, pH 7.5 containing 0.3 M NaCl, 30 mM EDTA), and then a final wash with low-salt buffer at 65°C for 1 h. Radioactive signals were visualized on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and signal intensities were quantified using the manufacturer's ImageQuant software.

Northern Blotting. Northern blot analysis was carried out using standard methods (Brown, 1997). The blots were probed with gene-specific antisense oligonucleotides (ON), as follows (intronic sequences are shown in lower case): ON-4, 5'-AAA-TTT-TCA-TGT-TCT-CAT-TGA-G-3', complementary to P450 2C6 cDNA; ON-28, 5'-TAG-CGG-CCC-TTG-GTC-ATC-AG-3, complementary to P450R cDNA nucleotides 1999–2019 (exon 16); ON-325, 5'-gcc-tca-ctt-ac-CTG-CGC-TCT-3', complementary to sequences at the junction between P450R exon 1 and intron 1; ON-327, 5'-Gca-cgg-ctt-ac-TTG-CTC-TCG-3', complementary to the junction between P450R exon 13 and intron 13 (G residue at 5'-end added to facilitate T4 kinase labeling of probe). Hybridization conditions were as described else-

where (Waxman, 1991) at 40°C without formamide in the case of probe ON-4, and at 50°C with 10% formamide in the case of probes ON-28, ON-325, and ON-327.

Results

T3 Induction of Nuclear P450R mRNA. Treatment of rats with a receptor-saturating dose of T3 (200 µg/100 g of BW) induces total liver P450R RNA by up to ~10-fold (Ram and Waxman, 1992). To determine whether this increase reflects nuclear, as opposed to cytoplasmic events, RNA was prepared from liver nuclei isolated 8, 12, or 24 h after T3 treatment. P450R RNA levels present in the nuclear RNA fraction were compared with those found in cytoplasmic RNA from the same liver samples. Northern blot analysis using an oligonucleotide probe specific for P450R exon 16 revealed a 4- to 5-fold increase in total P450R RNA that was first detected 12 h after T3 treatment. By contrast, T3 induction of nuclear P450R RNA was readily detected at the 8-h time point (Fig. 1A). Analysis of P450 2C6 RNA, which is not responsive to thyroid hormone treatment (Ram and Waxman, 1991), verified that this RNA and its nuclear precursor were not subject to T3 induction (Fig. 1C). The method presently used to isolate 'total liver RNA' yields cytoplasmic RNA free of unspliced nuclear precursors (Sundseth et al., 1992). This was verified by reprobating the Northern blot shown in Fig. 1A with an oligonucleotide probe that spans the junction between P450R exon 13 and intron 13 (Fig. 1B). This splice junction probe contains 10 nucleotides of exon 13 sequence followed by 10 nucleotides of intron 13 sequence. The 10-nucleotide exonic sequence is too short to hybridize to mature cytoplasmic P450R RNA, as evidenced by the absence of a signal in Fig. 1B, lanes 1 to 8. By contrast, unspliced P450R nuclear precursor RNA, in which the exon 13-intron 13 junction is preserved, readily hybridizes to this probe to give a pattern of T3 induction similar to that seen with the P450R exonic probe (lanes 9–16). A similar specificity for nuclear P450R RNA and a similar T3 induction pattern was observed using a P450R exon 1-intron 1 junction probe (Fig. 1D).

Thus, the strong induction of cytoplasmic P450R RNA by T3 is preceded by, and can be quantitatively justified, by a corresponding increase in P450R's primary nuclear transcript.

We previously reported that the induction of liver P450R RNA in T3-treated euthyroid rats is substantially reversed 24 to 36 h after hormone treatment, despite the continued elevation of plasma T3 levels (Ram and Waxman, 1992). In the experiment shown in Fig. 1, cytoplasmic P450R RNA was still elevated at 24 h (lanes 7 and 8); however, P450R nuclear precursor was decreased at this time to a level lower than that of untreated animals (lanes 15 and 16 versus lanes 9 and 10; integrity of P450 2C6 control hybridization signal in the reprobing shown in panel 1C). This suppression of nuclear P450R at 24 h was observed with several different hybridization probes for nuclear P450R transcripts (Fig. 1D), and in a separate series of experiments was shown to persist at 36 h (Fig. 2, C and D; first T3 injection time course). In this latter experiment, plasma T3 was still ~2.5- to 3-fold above the basal euthyroid level at 24 to 36 h, corresponding to a calculated 75 to 80% T3 receptor saturation (Table 2). This suggests that transcription of P450R nuclear precursor RNA, or its stability, is down-regulated as a secondary response to hyperthyroidism. Repeat injection of T3 at 24 h under conditions that give rise to a calculated ~97% T3 receptor occupancy (Table 2) did, however, stimulate a rise in nuclear P450R transcripts 12 h later. This rise was again followed by a secondary increase in cytoplasmic P450R mRNA (Fig. 2A), followed by a decrease in nuclear P450R RNA to below basal level at 24 h, with the overall pattern similar to that induced by the first T3 injection. Thus, the down-regulatory effects of moderately high T3 levels evident from the low 24 h nuclear P450R RNA signals can be overcome by reinfusion of a receptor-saturating T3 dose.

Enhanced P450R Transcription After T3 Treatment. To further elucidate the mechanism whereby T3 induces an increase in unspliced nuclear P450R RNA, we investigated whether T3 stimulates an increase in P450R transcription

TABLE 1

Oligonucleotides used to synthesize probes for nuclear run-on assays

Shown are the sense and antisense primers (top and bottom line of each pair, respectively) used in PCR reactions to synthesize the indicated exonic (E) and intronic (I) fragments of the P450R gene. Sizes obtained for each PCR fragment were in agreement with the calculated sizes shown in the Table, except as noted for intron 10.

Oligonucleotides	Product Location	Product Length	Primer Sequence
		<i>base pairs</i>	
ON 395/396	E2–E5	520 ^a	5'–gTTG–TAT–CAC–CAA–CAT–GGG–3' 5'–gAG–CAA–ACT–TGA–CCC–CAG–TGA–3'
ON 397/398	E6–E8	300 ^a	5'–GTA–TTT–GGT–CTT–GGG–AAC–AA–3' 5'–GGT–TTC–TGG–TTC–TCG–TAG–CT–3'
ON 352/326	I8	777	5'–GTG–AGT–GGA–GGA–AGC–GGG–GT–3' 5'–gAT–CGA–AGG–GGC–tga–tgg–gaa–3'
ON 352/353	I8–E10	1101	5'–GTG–AGT–GGA–GGA–AGC–GGG–GT–3' 5'–gCA–TCG–AGA–TTG–TTT–AGA–GAC–3'
ON 354/355	I10	~725 ^b	5'–GTA–AGT–TCC–AGA–ATT–GAG–GG–3' 5'–gCT–GCG–GCA–GGA–AAG–AAA–CAG–3'
ON 399/400	E10–E13	720 ^a	5'–GTA–TGA–ATC–TGG–AGA–TCA–CG–3' 5'–gCT–TGC–TCT–CGA–AGC–CAA–GCT–3'
ON 432/433	E11–E13	427 ^a	5'–gCGC–ACG–GCC–CTC–ACC–TAC–TAC–CTG–3' 5'–gCGC–GGC–CGC–CAT–TCT–CG–3'
ON 434/293	E13–E16	869 ^a	5'–gCCC–ATG–TTC–GTG–CGC–AAA–TCT–CAG–3' 5'–ACT–GAA–AAC–AAC–TTT–ATT–CC–3'
ON 356/293	I15–E16	628	5'–GTG–AGT–AAA–AGG–GAC–TGG–GG–3' 5'–ACT–GAA–AAC–AAC–TTT–ATT–CC–3'

^a Probe prepared by PCR of cloned P450R cDNA, and thus excludes intronic sequences.

^b Measured size of intron 10 fragment (~725 base pairs) was smaller than the 1-kilobase pair size previously estimated by restriction mapping (Porter et al., 1990).

initiation as opposed to an increase in nuclear P450R stability. Earlier studies had shown that T3 treatment of rats could elevate liver P450R transcription initiation measured in a run-on transcription assay, but only up to ~2-fold (O'Leary et al., 1997). In view of the large increase in P450R nuclear precursor RNA observed under the same conditions of T3 treatment (Fig. 1 and Fig. 2), we investigated whether the previous nuclear run-on assay (O'Leary et al., 1997), which employed full-length P450R cDNA as the filter-bound hybridization probe, may have underestimated the T3 transcriptional response. To evaluate this possibility, and to deter-

mine whether the P450R transcription rate in T3-stimulated liver is constant across the P450R gene, nuclear run-on studies were carried out using filter-bound hybridization probes corresponding to nine different fragments of the P450R gene. These fragments were synthesized by PCR and span a large portion of the P450R gene, ranging from an exon 2 to exon 16 (Table 1). Run-on transcription analysis revealed that T3 treatment increased the transcription rate across all nine P450R gene segments. Moreover, the increase in transcriptional rate (up to 6- to 8-fold; Fig. 3B) is sufficient to account for the observed induction of P450R nuclear precursor and cytoplasmic RNA. However, little or no P450R transcription induction was apparent when P450R cDNA was used as probe on the same filter (pCMV-Red; Fig. 3A), in agreement with our earlier report (O'Leary et al., 1997). This suggests that filter-bound radioactivity observed with the intact, cloned P450R cDNA may include background hybridization signals not accounted for in the pGEM2 plasmid negative control (compare Fig. 3A).

Processing and regulated degradation of nascent RNA transcripts is reported to occur in a time-dependent manner under conditions of the nuclear run-on assay (Tsai and Su, 1995). Accordingly, if RNA processing or changes in the rate of nuclear precursor RNA degradation contribute to the overall increase in liver nuclear P450R RNA in T3-treated rats, then we may expect to see a larger induction of nascent P450R RNA transcripts when assayed after a 45-min incubation compared with a 5-min nuclear run-on assay. Figure 4 shows, however, that P450R nuclear RNA transcription is elevated by T3 to the same extent when measured in a 5-min assay compared with a 45-min assay (lanes 1–3 versus lanes 4–6), with no statistically significant differences in the fold-induction between the two assay time points (data not shown). The absence of a dramatically higher induction at 45 min suggests that post-transcriptional nuclear events, such as a decrease in the rate of nuclear RNA degradation, do not contribute significantly to the observed increase in nuclear P450R RNA. The overall hybridization signals were weaker in the 5-min incubations because of the less extensive ^{32}P -label incorporation into nascent RNA at this time point. Figure 4 further shows that the T3-stimulated increase in P450R transcription is substantially reversed by 24 h (lanes 3 and 6), in agreement with the depletion of P450R nuclear RNA precursor seen at this same time point (Fig. 1). As a positive control, we monitored T3-stimulated run-on transcription of S14 RNA, which is a classic thyroid hormone-responsive liver mRNA (Jump, 1989; Goumaz et al., 1994), and found that it is induced by 3- to 4-fold in nuclear run-on assays carried for either 5 min or 45 min (Fig. 4 and data not shown). Moreover, in contrast to P450R transcription, the T3-stimulated rate of S14 RNA transcription was largely maintained at the 24-h time point.

Actinomycin D Inhibits T3-Stimulated P450R RNA Induction. We next examined whether the T3-stimulated increase in P450R transcriptional activity could be blocked by RNA polymerase inhibitors administered *in vivo*. In the experiment shown in Fig. 5, the RNA polymerase II inhibitor actinomycin D was administered to rats together with T3. Sixteen hours later the livers were removed and nuclei were isolated. Nuclear run-on analysis revealed an increase in transcription across the P450R gene, in agreement with Fig. 3. Moreover, this increase in transcription was blocked by

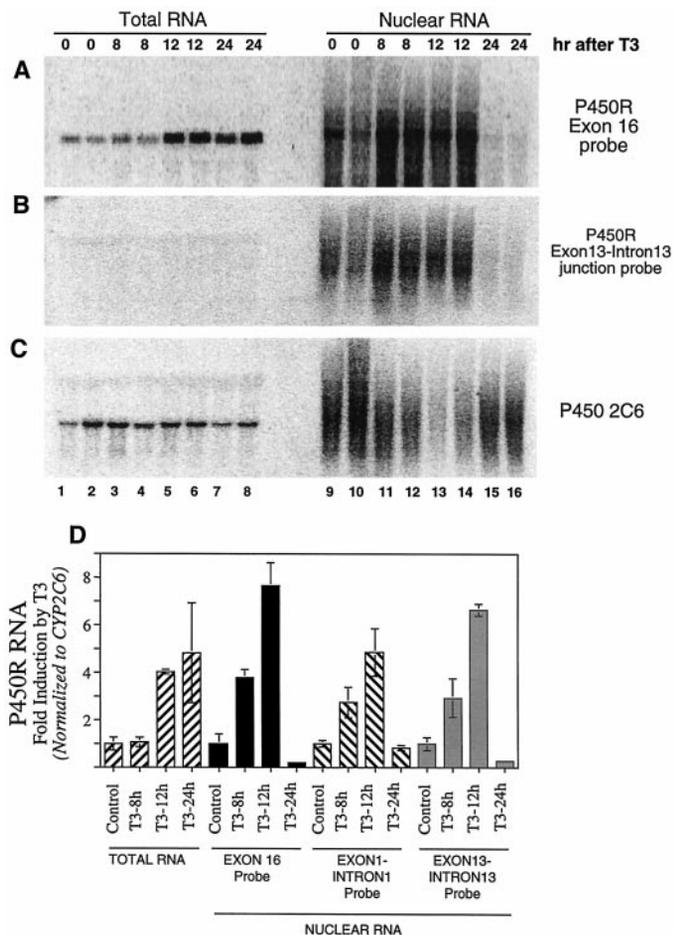


Fig. 1. Northern analysis of T3-induced rat liver nuclear RNA. Euthyroid rats were injected with a single injection of T3 (200 $\mu\text{g}/100$ g of BW, *i.p.*) and killed at the indicated times. Control rats received vehicle injection only and were killed 1 h later. Total RNA (20 μg) and nuclear RNA (15 μg) isolated from the same individual livers were fractionated on a 1.5% agarose gel and probed sequentially with single-stranded oligonucleotides complementary to and specific for P450R mRNA (A), the junction of exon 13 and intron 13 of P450R nuclear precursor RNA (B), and P450 2C6 mRNA (C). T3 induces nuclear P450R RNA 3- to 4-fold by 8 h (lanes 11 and 12 versus 9 and 10 of A and B; also see quantitative data in D). Similar results were obtained using an exon 1-intron 1 junction probe (D). This induction was followed by a similar increase in total cytoplasmic P450R mRNA, which first became apparent at 12 h (lanes 5 and 6). A near-complete suppression of nuclear P450R RNA is seen at the 24-h time point (lanes 15 and 16). Reprobing for P450 2C6 RNA, which is not T3-inducible (C, lanes 1–8), verified the presence of intact nuclear RNA in the 24-h samples. Lighter P450 2C6 RNA signals seen in lanes 12 to 14 reflect low RNA loadings. The suppression of nuclear P450R RNA 24 h after T3 injection seen in two individual rats in this experiment (lanes 15 and 16) was confirmed in three other independent experiments (e.g., see Fig. 2, below). Quantitative data for the fold-induction of total liver and nuclear P450R RNA after T3 treatment after normalization to P450 2C6 RNA levels are presented in D.

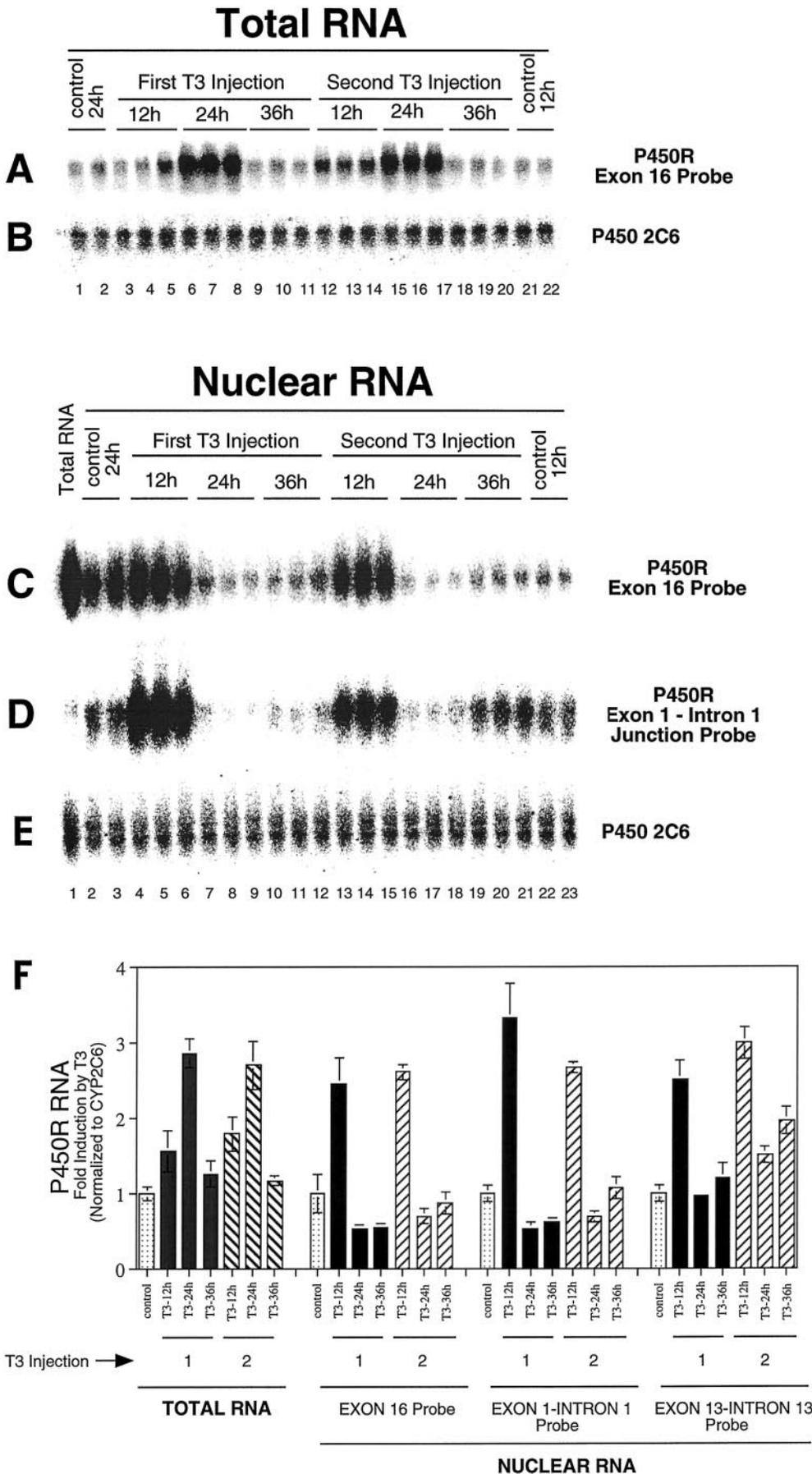


Fig. 2. Repeat induction of P450R nuclear and cytoplasmic RNA by sequential T3 injections. Euthyroid rats were given two injections of T3 (200 μ g/100 g of BW, i.p.) spaced 24 h apart. Rats [$n = 3$ per treatment group, except for the vehicle-treated control groups ($n = 2$ rats at 12 h and $n = 2$ at 24 h)] were killed 12 to 36 h after the first T3 injection, or 12 to 36 h after the second T3 injection, as indicated. Total RNA (i.e., cytoplasmic RNA) (A and B) and nuclear RNA fractions (panels C–E) were analyzed using hybridization probes as described in Fig. 1. Shown in lane 1 of panels C to E is a total liver RNA sample 12 h after the first T3 injection. Quantification of RNA hybridization signals by PhosphorImager analysis after normalization to P450 2C6 RNA is shown in F. Plasma T3 levels for the rats included in this experiment are shown in Table 2.

actinomycin D (Fig. 5B), consistent with the induction being a true RNA polymerase-dependent transcriptional event. This effect of actinomycin D reflects its inhibition of RNA polymerase in liver in vivo, and does not result from an in vitro inhibition of the nuclear run-on transcription assay itself by residual actinomycin D. This was evident from the fact that the in vivo actinomycin D treatment did not decrease the efficiency of ^{32}P label incorporation into total nascent nuclear RNA in the run-on transcription assay (data not shown). A similar inhibition of T3-stimulated P450R transcription was obtained in experiments in which rats were treated with T3 in combination with α -amanitin (0.3 mg/kg BW; Sigma) (Voigt et al., 1978), an inhibitor of RNA polymerases II and III (data not shown).

Discussion

Thyroid hormone plays an important role in P450R gene expression, as demonstrated by the ~80% decrease in P450R activity, protein, and mRNA levels in the hypothyroid state, and by the supraphysiological increase (up to ~10-fold) in hepatic P450R mRNA levels under conditions of hyperthyroidism (Ram and Waxman, 1992). In the present study, we examined the mechanisms that govern the large increase in hepatic P450R mRNA that is seen in rats rendered hyperthyroid by T3 treatment. Northern analysis of heterogeneous liver nuclear RNA revealed that T3 stimulates up to a 6- to 8-fold increase in unspliced nuclear P450R RNA. This increase, already apparent by 8 h, preceded a corresponding increase in cytoplasmic P450R mRNA that was first detected in the same liver samples at 12 h. This time course is consistent with the presence of a precursor-product relationship between the nuclear precursor and mature cytoplasmic P450R RNA. These effects of T3 on nuclear P450R RNA were apparent when using an exonic P450R probe and when using either an exon 1-intron 1 junction probe or an exon 13-intron 13 junction probe, both of which were shown to be specific for the unprocessed nuclear P450R precursor RNA.

The T3-stimulated increase in nuclear P450R RNA observed in the present study is distinctly greater than the ~2-fold increase in P450R run-on transcription rates reported previously (O'Leary et al., 1997). To address this dis-

crepancy, we carried out a detailed run-on transcriptional study using probes corresponding to nine individual seg-

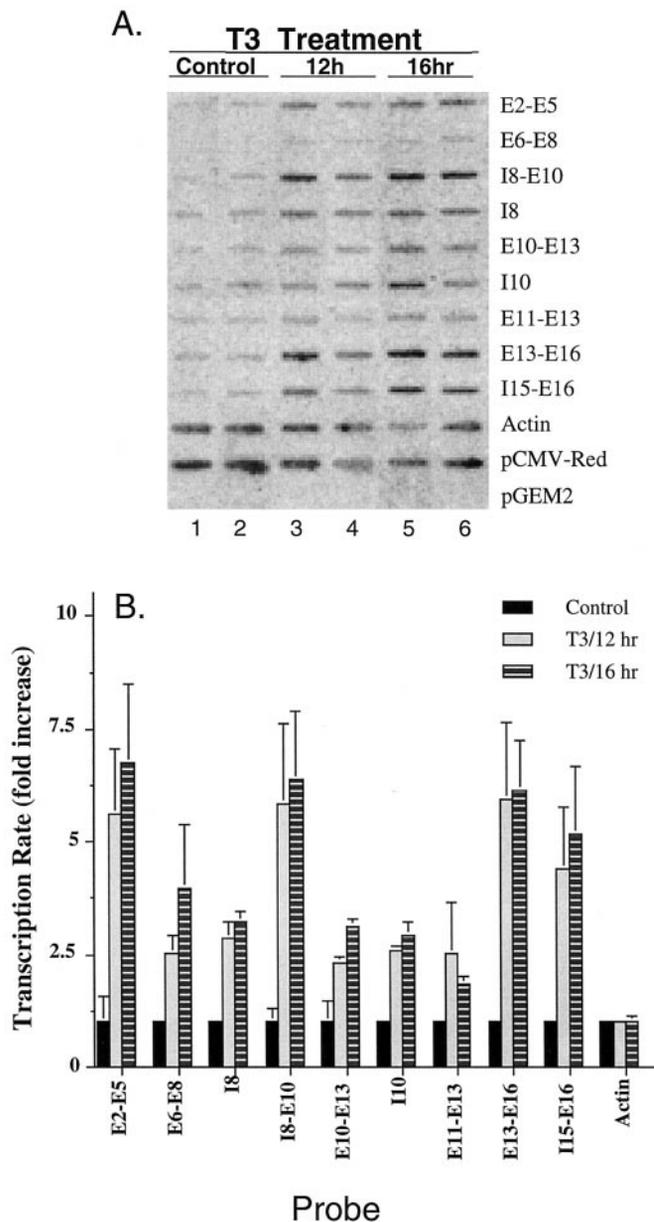


Fig. 3. T3 induction of P450R RNA: Nuclear run-on transcriptional analysis. Nuclei isolated from livers of rats that were untreated (control; lanes 1 and 2), or were treated with T3 for 12 h (lanes 3 and 4) or for 16 h (lanes 5 and 6) were incubated for 30 min at 30°C in the presence of [^{32}P]UTP to elongate nascent RNA transcripts. Transcription was terminated by placing the samples in ice-cold water. Labeled RNA was then isolated and hybridized to filters containing PCR products corresponding to the indicated exonic (E) and intronic (I) fragments of the P450R gene, or containing plasmid DNA encoding actin or P450R (pCMV-Red) or pGEM2 empty plasmid (Promega, Madison, WI), which served as a negative control. Shown is a PhosphorImager exposure of the hybridization signals (A), with quantification (mean \pm range of duplicates) expressed as fold-increase in hybridization signal intensity compared with control livers after subtracting pGEM2 (background) signals and normalizing the data to the actin controls (B). The lower fold-induction seen in this experiment with some of the probes (e.g., E6-E8, E11-E13) may reflect difficulties in accurately quantitating low basal hybridization signals. The lower fold-activation seen with probe I10 could reflect the same technical difficulties or may be caused by efficient processing and degradation of the corresponding intronic RNA sequence. Probes E6-E8 and E11-E13 are shorter than the other probes (compare Table 1), resulting in weaker hybridization signals.

TABLE 2

Plasma T3 levels after T3 treatment of euthyroid rats

Data are for the experiment shown in Fig. 2, using *n* rats per group. Vehicle-treated controls were killed 12 or 24 h after injection, as indicated. Plasma T3 levels of all six T3-treated rat groups were significantly higher than control (euthyroid) T3 levels ($p < 0.0001$; unpaired two-tailed *t* test). Nuclear occupancy of T3 receptors (*q*) was calculated from plasma T3 values according to Oppenheimer et al. (1977) using the equation $q = p/(p + p_{1/2})$, where *p* is the measured plasma T3 concentration, and $p_{1/2} = 0.67$ ng/ml, the plasma T3 concentration at which T3 nuclear receptors are 50% occupied (Oppenheimer et al., 1977).

Rat Treatment	<i>n</i>	T3 ng/ml	T3 Receptor Occupancy ^b %
Control (12 h)	2	0.80 \pm 0.08	54
Control (24 h)	2	0.87 \pm 0.16	56
First T3 Injection			
12 h	3	26.10 \pm 2.70	97
24 h	3	2.73 \pm 0.46	80
36 h	3	2.03 \pm 0.19	75
Second T3 Injection			
12 h	3	23.40 \pm 3.40	97
24 h	3	4.60 \pm 0.70	87
36 h	3	1.88 \pm 0.14	74

ments of the gene, spanning exon 2 to exon 16. With each exonic or intronic probe tested, T3 stimulated a substantial increase in P450R gene transcription. Increases as high as ~6- to 8-fold were obtained with four of the probes. The lower fold-induction seen with some of the probes could relate to inaccuracies intrinsic to the quantification of low basal hybridization signals; alternatively, it might reflect instability of some of the nascent intronic sequences. Additional regulatory steps, such as transcriptional pausing, are also possible [compare T3 regulation of rat liver apolipoprotein A-I transcription elongation (Lin-Lee et al., 1995)]. By contrast, when the P450R cDNA probe used previously (O'Leary et al., 1997) was employed in side-by-side nuclear run-on studies, little or no T3 induction of P450R transcription was evident in several, but not all, of our experiments (Figs. 3 and 4 versus Fig. 5; pCMV-Red). This finding suggests that background hybridization signals, not accounted for by the pGEM2 plasmid negative control, can contribute to a high "minus T3" hybridization signal in the case of the cDNA probe, and that this high background results in a low apparent fold-induction of the gene. Further confirmation that the T3-stimulated increase in P450R nuclear run-on activity presently reported reflects a genuine RNA polymerase-dependent transcriptional activation was provided by the demonstration that RNA polymerase inhibitors, administered *in vivo*, effectively block this increase (Fig. 5).

The induction of hepatic P450R mRNA in T3-treated rats is delayed by several hours relative to that of S14 mRNA (Ram and Waxman, 1992), a classic early thyroid hormone-responsive liver gene product (Jump, 1989; Goumaz et al., 1994). This suggests that the transcriptional activation of P450R is a secondary response to thyroid hormone. A functional thyroid response element with a classic direct repeat 4 (DR4) organization has, however, been identified at nucleotides -561 to -546 of the rat P450R gene and shown to confer up to 5-fold transcriptional activation by T3 in reporter gene assays (O'Leary et al., 1997). Accordingly, the delayed activation of P450R compared with S14 and other thyroid-responsive early liver gene products suggests that T3

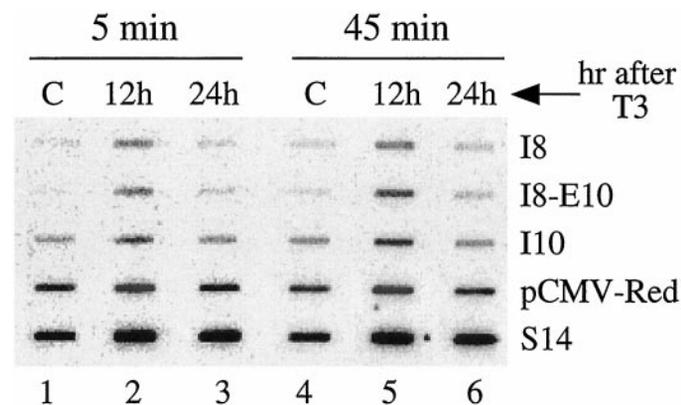


Fig. 4. Effect of run-on assay incubation time on extent of T3 induction. Nuclei isolated from untreated (control lanes 1 and 4), T3-treated for 12 h (lanes 2 and 5), and T3-treated for 24 h (lanes 3 and 6) rat livers were incubated in the presence of [α^{32} P]UTP to elongate nascent RNA transcripts at 30°C for 5 min (lanes 1–3) or 45 min (lanes 4–6). Isolation of labeled RNA, hybridization to the indicated P450R probes and visualization by PhosphorImager analysis were as described in Fig. 3. Twice as much 32 P-labeled nascent RNA was hybridized to the filters in lanes 4 to 6 compared with lanes 1 to 3.

induction of P450R transcription requires one or more thyroid hormone-inducible factors, in addition to the direct participation of thyroid hormone receptor.

T3-induced P450R mRNA returned to basal levels by 36 h after a single T3 injection given to euthyroid rats (Fig. 2). This decrease in cytoplasmic P450R mRNA was preceded by a reversal, to basal levels, of P450R transcription rates (Fig. 4) and nuclear R450R transcripts (Fig. 2). Indeed, unspliced nuclear P450R RNA was found to decrease to below basal levels 24 h after T3 treatment. These decreases in P450R RNA expression occurred despite the continued presence of T3 in plasma at a level 2.5- to 3-fold above normal euthyroid T3 levels (i.e., sufficient to saturate nuclear T3 receptors by an estimated 75–80%) (Table 2) (Oppenheimer et al., 1977). This suggests that hyperthyroidism may lead to a block in nuclear P450R RNA processing or may perhaps enhance degradation or increase export of the nuclear P450R RNA. T3 regulation of nuclear RNA processing and degradation have been reported for the apolipoprotein A-I and malic enzyme genes in rat liver (Song et al., 1988; Soyol et al., 1995). The down-regulation of nuclear P450R RNA expression, shown here to be a secondary response to hyperthyroidism, could

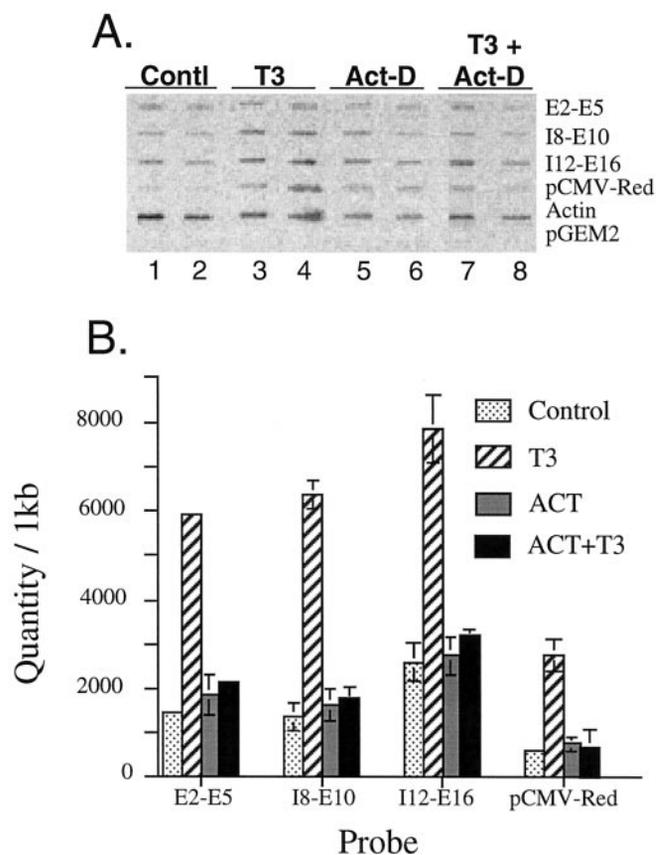


Fig. 5. Effect of actinomycin D on T3-induced P450R transcription. Liver nuclei isolated from rats that were untreated (lanes 1 and 2), T3-treated for 16 h (lanes 3 and 4), treated with actinomycin D for 16 h (lanes 5 and 6), or treated with T3 in combination with actinomycin D for 16 h (lanes 7 and 8) were incubated in the presence of [α^{32} P]UTP to elongate nascent RNA transcripts for 30 min at 30°C. 32 P-labeled RNA isolation, hybridization to a filter containing the P450R gene fragments and plasmid probes indicated, and data analysis were as described in Fig. 3. A, a PhosphorImager exposure of the hybridization signals; B, quantity of radioactivity (mean \pm range of duplicates) per kilobase pair of probe, normalized to the actin control signal intensity of each sample.

serve as a mechanism for the liver to maintain P450R levels within physiologically acceptable limits. Potential mechanisms for this apparent down-regulation of P450R gene transcription under conditions of prolonged T3 stimulation include down-regulation of thyroid hormone receptor, thyroid hormone receptor-associated proteins (Fondell et al., 1999) or the receptor's heterodimerization partner, retinoid X-receptor (Mano et al., 1994). Additional mechanisms may include induction or activation of transcriptional inhibitors, such as the nuclear receptor corepressor (Chen and Li, 1998) or poly(ATP-ribose) polymerase, which was recently shown to associate with, and thereby inhibit, transcriptional responses directed by DNA- and ligand-bound thyroid hormone receptor (Miyamoto et al., 1999).

Although the magnitude of the P450R transcriptional activation described here is sufficient to account for the observed T3-stimulated increases in P450R nuclear and cytoplasmic RNA, postnuclear events may additionally contribute to thyroid hormone regulation of this gene. Processes such as mRNA stability, 3'-polyadenylation, and mRNA translation are all known targets for thyroid hormone regulation (Krane et al., 1991; Murphy et al., 1992; Kern et al., 1996). One or more of these regulatory mechanisms may characterize P450R; for example, altered translational regulation is suggested by the marked discrepancy between P450R mRNA versus P450R protein and activity in hyperthyroid rat liver (Ram and Waxman, 1992). Further investigation will be required to elucidate fully the complex regulatory mechanisms through which thyroid hormone regulates expression of P450R in response to physiological and pathophysiological changes in thyroid hormone status.

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