

Post-Transcriptional Regulation of Hepatic NADPH-Cytochrome P450 Reductase by Thyroid Hormone: Independent Effects on Poly(A) Tail Length and mRNA Stability

DONGXU LIU¹ and DAVID J. WAXMAN

Division of Cell and Molecular Biology, Department of Biology, Boston University, Boston, Massachusetts

Received November 6, 2001; accepted January 22, 2002

This article is available online at <http://molpharm.aspetjournals.org>

ABSTRACT

Thyroid hormone [triiodothyronine (T3)] positively regulates NADPH cytochrome P450 reductase (P450R) mRNA expression in rat liver, with P450R transcription initiation being a key regulated step. T3 is presently shown to have significant post-transcriptional effects on P450R expression. T3 increased the size of cytoplasmic P450R mRNA by ~105 nucleotides 12 h after T3 treatment, followed by a return to basal levels at 24 h. Primer extension analysis and Northern hybridization with 5'-untranslated region probes revealed no change in P450R mRNA 5' structure with T3 treatment. By contrast, RNase H analysis revealed a transient, T3-induced increase in P450R mRNA poly(A) tail, from ~100 to ~205 A. This increase in P450R polyadenylation, detectable in the nucleus 8 h after T3 treatment and in the cytoplasm at 12 h, was transient and was

reversed by 16 h, when the T3-induced accumulation of cytoplasmic P450R mRNA was near maximal. Actinomycin D blocked the increase in P450R poly(A) tail and the induction of P450R mRNA, indicating a requirement for ongoing gene transcription for both T3 responses. T3 treatment destabilized P450R mRNA in rat liver *in vivo*, as shown by the T3-dependent 6-fold decrease in cytoplasmic P450R mRNA half-life, from a basal value of ≥ 16 h in uninduced liver to ~2.5 h, measured 24 h after T3 administration. These findings demonstrate that T3 increases nuclear polyadenylation of P450R RNA as a transient, early regulatory response and that this response is temporally dissociated from the subsequent decrease in cytoplasmic P450R mRNA stability.

NADPH cytochrome P450 oxidoreductase (P450R) is a ubiquitous microsomal flavoprotein that is essential for the transfer of electrons from NADPH to microsomal cytochrome P450 (P450) (Porter, 1991; Strobel et al., 1995). P450R is an obligatory, often rate-limiting factor in microsomal cytochrome P450-linked oxidative metabolism of drugs, chemical carcinogens, and endogenous steroidal substrates (Kaminsky and Guengerich, 1985; Waxman et al., 1989; Cawley et al., 1995). P450R also plays a critical role in P450-independent metabolic processes, including reductive metabolism leading to 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 may contribute to the cell's adaptive response to oxidative stress (Landriscina et al., 1988; Chen and Cederbaum, 1997; Choi et al., 1997). P450R is encoded by a single gene, which spans more than 50 kb and contains 16 exons (Porter et al., 1990). The first exon, com-

prised of 56 nt of untranslated sequence, has been localized to a segment of DNA 30.5 kb upstream of the coding exons (O'Leary et al., 1994). In the case of the mouse P450R gene, an alternate 5' exon is used in a tissue-specific manner (O'Leary et al., 2000).

P450R is most highly expressed in liver, where it is regulated by xenochemicals and by endogenous hormones, including thyroid hormone, T3. Liver P450R is dependent on normal thyroid hormone levels for expression, as shown by the $\geq 75\%$ decrease in liver P450R mRNA, protein, and activity in T3-deficient rats (Waxman et al., 1989; Ram and Waxman, 1992). Physiological replacement of T3 but not other pituitary-dependent hormones substantially restores P450R levels in hypophysectomized or hypothyroid rats, indicating that T3 is required for full expression of P450R activity. In contrast, T3 treatment of euthyroid rats stimulates up to a 10-fold increase in P450R mRNA without significantly affecting P450R protein or activity (Ram and Waxman, 1992). Further studies support the conclusion that within 8 to 12 h, the T3 stimulates increases in P450R transcription and corresponding increases in unspliced nuclear P450R RNA levels, followed by an increase in cytoplasmic P450R mRNA (Li et

Supported in part by National Institutes of Health grant R01-DK53767 (to D.J.W.).

¹ Present address: Center for Blood Research, Harvard Medical School, Boston MA 02215.

ABBREVIATIONS: P450R, NADPH-cytochrome P450 reductase; P450, cytochrome P450; kb, kilobase(s); T3, triiodothyronine; ON, oligonucleotide; UTR, untranslated region; nt, nucleotide(s); bp, base pair(s); PCR, polymerase chain reaction.

al., 2001). Studies of the mechanisms by which T3 regulates *P450R* transcription have identified a functional thyroid response element in the *P450R* promoter and have shown it to be composed of an imperfect repeat of the classic thyroid response motif AGGTCA (O'Leary et al., 1997).

Although the transcriptional mechanisms by which T3 regulates *P450R* gene transcription are fairly well understood, much less is known about potential post-transcriptional events that contribute to the regulation of *P450R* expression by thyroid hormone. The present study addresses this issue and investigates the effects of T3 on the polyadenylation of *P450R* RNA. In addition, the effects of T3 on the stability of *P450R* mRNA in liver in vivo are examined. T3 is shown to induce a discrete increase in the length of the *P450R* RNA poly(A) tail by a mechanism involving increased nuclear polyadenylation. This increase is temporally distinct from the subsequent decrease in *P450R* mRNA stability seen in T3-induced rat liver. These findings are discussed in the context of the post-transcriptional mechanisms of thyroid hormone action.

Materials and Methods

Animals. Adult male Fischer 344 rats, housed under standardized conditions of light and temperature, were given a single i.p. injection of T3 (Sigma, St. Louis, MO) at a receptor-saturating dose (200 μ g of T3/100 g of body weight) using 0.01 M KPi and 0.9% NaCl buffer, pH 8.3, as vehicle (Li et al., 2001). Animal experiments were carried out two to three times and included at least two individual animals per time point or treatment group. Replicates shown in each figure correspond to separate animals, with each liver sample processed separately. In some experiments, euthyroid rats were treated with the RNA polymerase inhibitor actinomycin D (Sigma) using established methods (Connor et al., 1996). Actinomycin D was given at 0.25 mg/100 g of body weight, i.p. (using 0.1 M KPi buffer, pH 8, as vehicle), either concurrent with T3 treatment or 12 or 24 h after T3 treatment, as specified. Control rats received the same volume of vehicle. No adverse health effects of actinomycin D were observed over the course of these experiments.

RNA Isolation. Total cytoplasmic RNA was isolated from rat liver by extraction according to Chomczynski and Sacchi (1987) using guanidine isothiocyanate solution (Invitrogen, Carlsbad, CA) (Waxman, 1991). This RNA isolation procedure yields cytoplasmic RNA, with little if any unspliced nuclear precursor RNA detectable (Sundseth et al., 1992). Nuclei were prepared from fresh rat liver and stored in liquid nitrogen (Sundseth et al., 1992). Nuclear RNA was isolated from the frozen nuclei using a CsCl (optical grade, Invitrogen) purification method (Kingston et al., 1996). In brief, $\sim 10^7$ nuclei were homogenized in buffer D [4 M guanidinium isothiocyanate, 0.1 M Tris-Cl, pH 7.5, and 1% (v/v) 2-mercaptoethanol]. Sodium lauryl sarcosinate [20% (w/v) stock] was added to give a final concentration of 0.5%, and 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) and centrifuged at 20°C overnight at 100,000g. RNA pellets were dissolved in diethyl pyrocarbonate-treated water and stored at -70°C . Poly(A) mRNA was isolated from total RNA by oligo(dT) cellulose chromatography using Oligotex mRNA Midi Kit (QIAGEN, Valencia, CA) following the manufacturer's protocol.

Northern Blotting. Total liver RNA (20 μ g per lane) was denatured and separated on 1.0% (w/v) agarose gels containing 18% (v/v) formaldehyde, transferred, and then UV cross-linked to nylon membranes (Brown, 1997). Antisense oligonucleotides (ON) were labeled with [γ - ^{32}P]ATP (6000 Ci/mmol, PerkinElmer Life Sciences, Boston, MA). Prehybridization (3 h) and hybridization overnight with ^{32}P -labeled oligonucleotide probes were carried out at the hybridization

temperature and in the presence of formamide concentrations calculated based on the length and GC composition of each probe (Waxman, 1991). The probes and hybridization conditions used were as follows: 5'-GTA-GCG-GCC-CTT-GGT-CAT-CAG-3' (probe ON-680, complementary to *P450R* exon 16; *P450R* cDNA nt 2036–2056; 50°C and 15% formamide); 5'-gCG-CGG-TCC-TGT-AGG-TCTC-3' (ON-754, complementary to *P450R* exon 1, nt 2–19; 50°C and 13% formamide); and 5'-gCC-TGC-GCT-CTC-CGG-TAC-CGA-CCA-GATC-3' [ON-318, complementary to *P450R* cDNA nt 1–27 (3' end of *P450R* exon 1); 50°C and 30% formamide]. Nucleotides are numbered according to the rat *P450R* cDNA sequence (GenBank accession M12516; RATCYPRM), unless indicated otherwise. This cDNA is missing the first 27 nt of *P450R* exon 1 (O'Leary et al., 1994). Probing for *P450* 2C6 RNA, which is not altered by T3 treatment, was performed as described previously (Waxman, 1991). Washed membranes were processed using standard procedures (Waxman, 1991). Autoradiographs were analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Poly(A) mRNA (5 μ g per lane) was analyzed by Northern blotting on high-resolution gels [1.3% (w/v) agarose gels containing 18% formaldehyde and electrophoresed at 30 V for 16 h] (Stoeckle and Guan, 1993). Individual lanes shown on each gel (Figs. 2 and 4–6, below) correspond to RNA samples isolated from individual livers within each animal treatment group.

RNase H Assay. Total liver cytoplasmic RNA or nuclear RNA was digested with RNase H (USB Corporation, Cleveland, OH) in the presence of either one or two oligonucleotide probes complementary to a specific sequence of the *P450R* 3'-UTR, as shown schematically in Fig. 1. This method, used to determine *P450R* mRNA poly(A) tail length, is a modification of an oligonucleotide-directed RNase H digestion assay described elsewhere (Chkheidze et al., 1999). In brief, 40 μ g of RNA was denatured for 5 min at 65°C and hybridized for 1 h at room temperature with *P450R*-specific 3'-UTR antisense probe ON-338 (5'-GCT-GCC-TGA-TAC-AAT-GGA-CC-3'; *P450R* cDNA nt 2231–2250) and/or ON-293 (5'-ACT-GAA-AAC-AAC-TTT-ATT-CC-3'; cDNA nt 2417–2436). Samples were then incubated for 1 h at 37°C with 2 U of RNase H in RNase buffer (20 mM Tris-HCl, pH 7.5, 20 mM KCl, 10 mM MgCl₂, 0.1 mM EDTA, and 0.1 mM dithiothreitol). RNA fragments were separated on 1% (w/v) agarose gels containing 18% (v/v) formaldehyde. RNAs were transferred and UV cross-linked to nylon membranes for Northern blotting using ^{32}P -labeled *P450R* antisense probe ON-329 (5'-GAGG-ACC-TAA-GGC-ACC-CATC-3'; cDNA nt 2297–2316). This method does not provide for the quantitative recovery of the released RNA fragments analyzed on the Northern blot.

Poly(A) Tail-Length Measurements. *P450R* 3'-UTR fragments released by RNase H digestion were sized on Northern blots based on

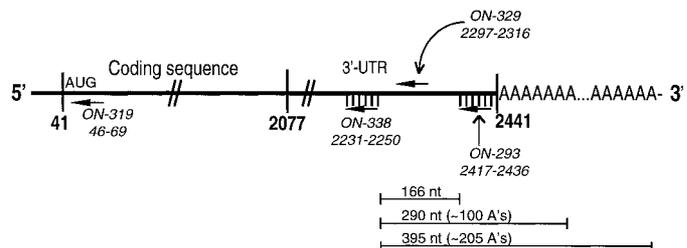


Fig. 1. Schematic diagram of *P450R* mRNA, indicating oligonucleotide probes (ON) used to characterize *P450R* mRNA by primer extension and RNase H assay. Antisense probe ON-319 (exon 2; nt 46–69) was used to detect the 5' end of *P450R* mRNA by primer extension (Fig. 3, below). RNase H digestion experiments (Figs. 4–6, below) were carried out in the presence of ON-338 alone or ON-338 + ON-293 to cleave the resultant DNA-RNA hybrids, indicated by hatched bars. The released 3'-UTR RNA fragments were subject to analysis of Northern blots probed with ON-329 (antisense nt 2297–2316). Shown at the bottom right are the sizes of the various fragments released by RNase H digestion of *P450R* (compare Fig. 4, below), with the poly(A) tail lengths as indicated. Nucleotide positions shown correspond to those of *P450R* cDNA, which is missing the 27 nt from the 5' of exon 1. Figure is not drawn to scale.

their relative migration in comparison with P450R RNA sizes standards. Size standards were prepared by PCR of P450R cDNA using a 5' primer containing a T7 RNA polymerase promoter sequence, followed by in vitro transcription using T7 RNA polymerase (Promega, Madison, WI). Primers were designed to generate P450R RNA fragments with lengths of 198 bp (ON-299 with ON-320), 267 bp (ON-298 with ON-320), and 358 bp (ON-290 with ON-293) (T7 RNA polymerase site indicated in lower case): sense, ON-299: 5'-taatacagactactatagggATGGGG-GACTCTCACGAAGA-3'; antisense, ON-320: 5'-gCTCTTTGACGGGTGGGGCCG-3'; sense, ON-298: 5'-taatacagactactatagggAGAGACCTACAGGACCGCGG-3'; sense, ON-290: 5'-taatacagactactatagggGAGCTACCAGCCTCCACCC-3'; and antisense, ON-293: 5'-ACTGAAAACAACCTTTATTCC-3'.

PCR reactions were performed for 30 cycles with *Taq* polymerase at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. An initial denaturation step (5 min at 94°C) and a final extension step (10 min at 72°C) were included. PCR fragments of the expected sizes were transcribed in vitro with 1 U of T7 RNA polymerase. Products were 5'-labeled with [γ -³²P]ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA), analyzed on 1% (w/v) agarose gels containing 18% (v/v) formaldehyde, transferred to membranes, visualized by PhosphorImaging, and analyzed using ImageQuant software.

Primer Extension. For primer extension assays (O'Leary et al., 1994), a 24-bp antisense primer complementary to a site downstream of the translation start site of P450R exon 2 (ON-319: 5'-GCA-CTG-TGT-TCT-TCG-TGA-GAG-TCC-3'; cDNA nt 46–69) (Fig. 1) was hybridized at 65°C for 90 min to 10 μ g of total liver RNA in hybridization buffer (20 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, and 100 mM NaCl). Samples were cooled at room temperature for 30 min and then reverse-transcribed for 60 min at 42°C with 5 U of murine leukemia virus reverse transcriptase (Stratagene, La Jolla, CA), dNTP mixture containing the fluorescently-tagged dye dUTP-TAMRA (Applied Biosystems, Foster City, CA) and RNase inhibitor (Promega). Reaction products were analyzed on 4.5% acrylamide, 6 M urea, 1 \times Tris/borate/EDTA gels using an AB1377 DNA sequencer (Applied Biosystems) in combination with the Genescex and Genotyper software by measuring fluorescence excitation at 633 nm and emission at 670 nm.

Results

T3 Treatment Increases the Size of Liver P450R mRNA. Rats were treated with a receptor-saturating dose of T3 and then killed either 12 or 24 h later. Liver poly(A) RNA was isolated and analyzed on high resolution Northern blots probed with an antisense oligonucleotide complementary to P450R exon 16. A small but consistent increase in the size of liver P450R mRNA was seen 12 h after T3 treatment (Fig. 2, lanes 3 and 4 versus lanes 1 and 2). This size increase was reversed 24 h after T3 administration (lanes 5 and 6), despite the continued increase in the overall abundance of P450R mRNA from 12 to 24 h (compare lanes 3 and 4).

Mouse P450R mRNA can be transcribed from two alternative promoters in a tissue-specific manner, one of which is preferentially used in untreated liver (O'Leary et al., 2001). We investigated, therefore, whether the longer P450 mRNA seen in T3-treated rat liver (Fig. 2) may reflect T3-stimulated transcription from a second promoter, giving rise to a transcript that contains a distinct, longer 5'-untranslated exon (exon 1a). Two approaches were taken to investigate this possibility. First, Northern blotting of control and T3-induced rat liver RNA was carried out using an exon 1 oligonucleotide probe. T3 induced a comparable increase in P450R RNA, independent of whether the blot was probed with an exon 1

probe (5.3-fold induction of P450R mRNA 12 h after T3 treatment; probe ON-318) or an exon 16 probe (5.2-fold induction; probe ON-680). Attempts to detect liver P450R mRNA by RT-PCR or by Northern blotting using a hybridization probe complementary to the sequence of mouse P450R exon 1a were negative (data not shown). Second, primer extension analysis using an exon 2 probe (ON-319; Fig. 1) revealed the same transcription start site for P450R in both uninduced and T3-induced liver samples (Fig. 3, A versus B and C). We conclude that alterations in the 5'-UTR of P450R mRNA are not likely to account for the longer mRNA seen 12 h after T3 treatment.

RNase H Analysis of P450R 3'-UTR. Next, we used an RNase H digestion protocol to investigate whether T3 induces changes at the 3' end of P450R mRNA. Uninduced and T3-induced liver RNA samples were hybridized to an oligonucleotide probe designed to direct RNase H cleavage to the corresponding P450R 3'-UTR RNA-DNA hybrid (probe ON-338; Fig. 1). Cleaved 3'-UTR RNA fragments were analyzed on a Northern blot hybridized with a probe complementary to a P450R sequence downstream of the RNase H cleavage site (probe ON-329; Fig. 1). Figure 4A shows that the 3'-UTR fragment released by RNase H digestion is distinctly longer, by ~105 nt, in the 12-h T3-treated liver RNA samples than in liver samples from untreated rats (395-nt fragment in lanes 7 and 8 versus 290-nt fragment in lanes 1 and 2; also see Fig. 1). Interestingly, the longer liver RNA transcript was absent from rats treated with T3 for shorter (4 or 8 h) or for longer periods of time (16 h). This size difference in P450R mRNA was associated with P450R sequences beyond nt 2436 [i.e., 5 nt upstream of the poly(A) tail], as was made evident by the elimination of the size difference when the liberated 3'-UTR fragment was generated in RNase H cleavage assays where probes ON-338 and ON-293 (nt 2417–2436) were both hybridized to P450R mRNA (Fig. 4A, lanes 11 and 12; "tail-cut-

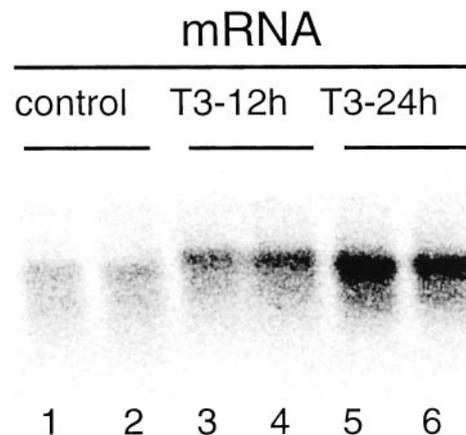


Fig. 2. Increase in size of P450R mRNA after T3 treatment. Poly(A)+mRNA was isolated from total cytoplasmic liver RNA prepared from vehicle control-treated rats or from rats given a single injection of T3 (200 mg/100 g body weight, i.p.) and killed 12 or 24 h later, as indicated. mRNA (5 μ g) was denatured and separated on a 1.3% (w/v) agarose/18% formaldehyde gel electrophoresed at 30 V for 16 h. P450R mRNA in the 12-h T3-treated livers (lanes 3 and 4) migrated more slowly than P450R mRNA isolated from the control livers (lanes 1 and 2) or the 24-h T3-treated livers (lanes 5 and 6). Northern blot was probed with ON-680, complementary to P450R exon 16. RNAs were ~2.5 kb in length, based on comparison with 18S and 28S RNAs in separate samples. PhosphorImaging analysis indicated T3-induced increases of 1.7- and 2.8-fold for P450R mRNA at 12 and 24 h, respectively, in comparison with lanes 1 and 2.

off" samples; released fragment 166 nt in length, corresponding to P450R mRNA nt 2251–2416; also see Fig. 1 and Fig. 5, lanes 13 and 14 versus lanes 11 and 12).

To ascertain whether the discrete T3-induced 3'-UTR size increase reflects an increase in poly(A) tail length as opposed to an alternate splicing event, RNase H digestion was carried out in the presence of oligo(dT)₁₅. RNase H treatment of liver RNA in the presence of oligo-dT led to a slight shortening of full-length P450R RNA (Fig. 4B, lanes 4–6 versus lanes 1–3). Moreover, RNase H digestion of 12-h T3-induced liver RNA in the presence of oligo(dT) + ON-338 (lane 11 versus lane 10) eliminated the substantial size difference that was seen in the presence of ON-338 alone (lane 8 versus lane 7). The resultant fragment (lanes 10–12) was slightly shorter than the fragment formed by RNase H digestion in the presence of ON-338 + ON-293 (lanes 13–15). These data are consistent with T3 stimulating the lengthening of P450R's poly(A) tail by ~105 nt, from ~100 A in the absence of T3 to ~205 A 12 h after hormone injection.

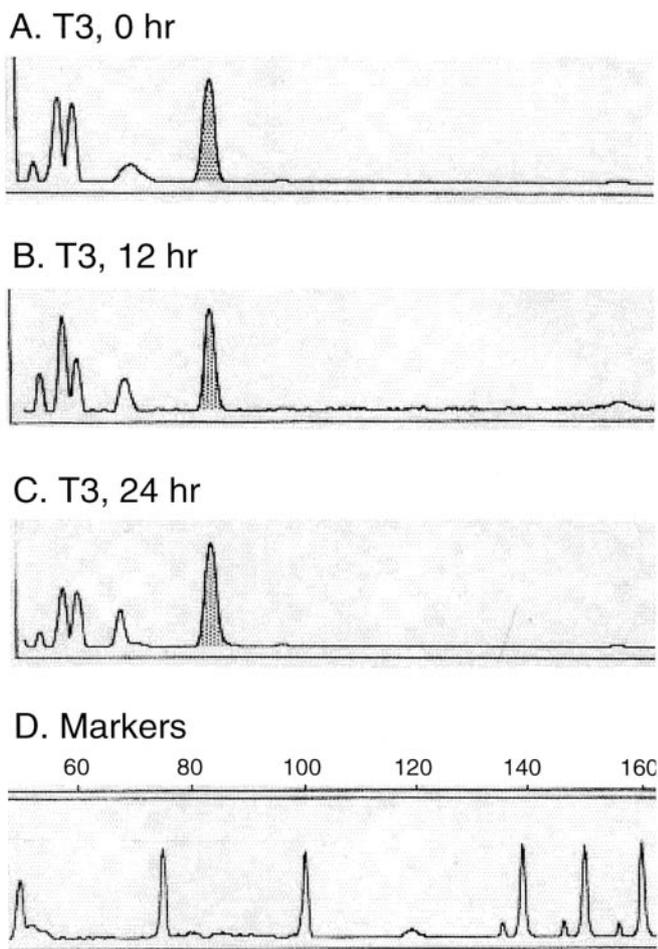


Fig. 3. Primer extension analysis of 5'-UTR of P450R mRNA. Total cytoplasmic RNA from untreated or from 12- or 24-h T3-treated rat liver (A, B, and C, respectively) was analyzed by primer extension. Antisense probe ON-319 (Fig. 1) was hybridized to liver RNA, followed by reverse transcription in the presence of a dNTP mixture containing fluorescently-labeled dUTP, as described under *Materials and Methods*. Images of the fluorescence dye-labeled products were analyzed as described under *Materials and Methods*. Standard markers (75, 100, 139, 150, and 160 bp) were analyzed in parallel (D). No size difference in the length of the 5' exon 1-containing primer extension product (shaded peak) was seen when comparing untreated with 12- or 24-h T3-treated P450R mRNA.

T3 Stimulates Nuclear Polyadenylation of P450R RNA in a Transcription-Dependent Manner.

Next, we investigated whether the effects of T3 described above reflect an increase in nuclear polyadenylation or a decrease in cytoplasmic deadenylation of P450R mRNA. RNase H analysis of nuclear RNA samples prepared from the same set of livers examined in Fig. 4 revealed that a P450R transcript with a longer poly(A) tail was present in the nucleus 8 h after T3 injection (Fig. 5, lanes 5 and 6 versus lanes 1 and 2). The longer nuclear P450R transcript was no longer detectable at

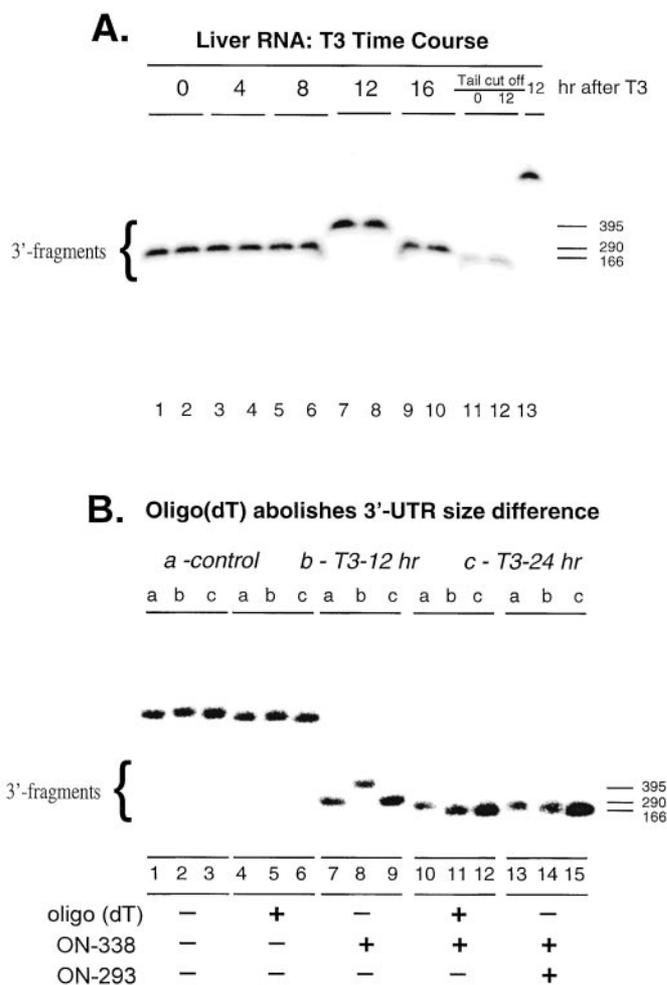


Fig. 4. RNase H analysis of cytoplasmic P450R mRNA poly(A) tail. T3-induced P450R mRNA poly(A) tail length was analyzed after RNase H digestion using probe ON-338 (nt 2231–2250) to form an RNA-DNA hybrid. Shown are liver cytoplasmic RNA samples from rats treated with T3 for 4 to 24 h, as marked. RNase H-released 3'-UTR fragments were electrophoresed, detected by hybridization to probe ON-329 (Fig. 1), and visualized by PhosphorImaging analysis. A 3'-UTR fragment of ~395 nt was evident in the T3-12-h liver cytoplasmic RNA samples (A, lanes 7 and 8). The P450R mRNA fragments shown correspond in size to ~166, 290, and 395 nt based on comparisons with authentic RNA size markers (see *Materials and Methods*). The 395-nt fragment corresponds in size to P450R mRNA 3'-UTR nt 2251–2441 (191 nt) plus 204 As; the 290-nt fragment to the same 191 P450 3'-UTR nt, plus ~99 As (i.e., a poly(A) tail length shorter by ~105 A than that of the 395 nt fragment); and the 166 tail-cut-off fragment corresponds to P450R mRNA 3'-UTR nt 2251–2416 (compare Fig. 1). A, lane 13, 12-h T3-induced total RNA sample without RNase H digestion. B, RNase H digestion in the absence of hybridizing oligonucleotides (lanes 1–3), with oligo(dT) alone, which results in a slightly shortened P450R mRNA (lanes 4–6), or in the presence of oligo(dT), ON-338, and ON-293 in combinations as indicated (lanes 7–15). The small difference in apparent band migration between lane 10 and lanes 11 and 12 was not reproducible in other experiments.

12 h (lanes 7 and 8), at which time it first became detectable in the cytoplasmic RNA fraction (Fig. 4A). In this animal model, T3 induction of liver nuclear P450R RNA is first seen at ~8 h and precedes the induction of cytoplasmic P450R RNA, which is detectable at 12 h and becomes maximal at 16 to 24 h (Li et al., 2001). We conclude that T3 stimulates an increase in nuclear P450R RNA polyadenylation at 8 h, with the resultant longer nuclear P450R RNA subsequently exported to the cytoplasm, where it is detected at 12 h.

To ascertain whether ongoing gene transcription is required for the T3-induced nuclear adenylation of P450R mRNA, rats were treated with the RNA polymerase inhibitor actinomycin D. Figure 6A shows that actinomycin D is effective as a transcriptional inhibitor when administered to rats in vivo, insofar as it fully blocks the T3-stimulated increase in cytoplasmic P450R RNA seen at 24 h (lanes 13 and 14 versus lanes 7 and 8; also see quantitation in Fig. 6C). Analysis of the nuclear RNA fraction also revealed a substantial block by actinomycin D of T3-induced nuclear P450R RNA synthesis (Fig. 6, B, lanes 9–12 versus lanes 3–6, and C). This block by actinomycin D in T3-stimulated P450R transcription abolished the increase in P450R poly(A) tail length, as revealed by RNase H analysis of both nuclear (Fig. 6D, lanes 3 and 4 versus lane 7) and cytoplasmic RNA samples (Fig. 6E, lanes 10 and 11 versus lanes 4 and 5). Thus, ongoing transcription is required for T3 to induce the poly(A) tail lengthening of P450R mRNA.

T3 Decreases Half-Life of P450R mRNA. Poly(A) tail length is a critical factor that regulates the stability of many eukaryotic mRNAs. Accordingly, we investigated whether T3 treatment alters the stability of liver P450R mRNA. The low abundance of liver P450R mRNA, estimated to be ~0.02% of translatable liver mRNA (Gonzalez and Kasper, 1981), precluded an analysis of P450R mRNA half-life by direct RNA labeling in vivo using a pulse-chase protocol. We therefore assayed the rate of liver P450R RNA decay after treatment of rats with actinomycin D under conditions that block *P450R* gene transcription (compare Fig. 6). Actinomycin was administered 24 h after a single injection of T3 or vehicle control, at which time cytoplasmic P450R mRNA was maximally induced. Northern analysis of liver RNA samples prepared at various times after actinomycin D treatment revealed a >6-fold more rapid decline of the T3-induced P450R mRNA ($t_{1/2}$

~ 2.5 h) compared with the uninduced mRNA ($t_{1/2} \geq 16$ h) (Fig. 7). In a separate series of experiments, actinomycin D was administered 12 h after T3 injection, at which time nuclear P450R RNA was maximally induced. Nuclear P450R RNA analyzed on Northern blots exhibited a $t_{1/2}$ of ~5 h (Fig. 7), a value that is intermediate between the values determined for uninduced and T3-induced cytoplasmic P450R RNA.

Discussion

In addition to its well-established nuclear receptor-dependent transcriptional effects, thyroid hormone regulates gene expression at a post-transcriptional level. Targets of this post-transcriptional regulation include the genes encoding growth hormone, α -myosin heavy chain, and retinoid X receptor, which are post-transcriptionally regulated by T3 in pituitary, heart, and liver, respectively (Murphy et al., 1992; Mano et al., 1994; Sindhvani et al., 1994; for review, see Williams et al., 1993). The present studies establish that hepatic *P450R* is also regulated by T3 post-transcriptionally, with regulation apparent at two distinct levels, nuclear polyadenylation and mRNA destabilization. Polyadenylation of nascent mRNAs is catalyzed by a nuclear poly(A) polymerase, followed by assembly into ribonucleoparticles and export to the cytoplasm, where further processing at the 3' end, including both deadenylation and re-adenylation can occur (Manley, 1995; Minvielle-Sebastia and Keller, 1999). The present finding that T3-stimulated P450R RNA polyadenylation was first detected in the nucleus 8 h after T3 treatment, and subsequently in the cytoplasm, at 12 h, indicates that T3 regulates the nuclear polyadenylation reaction, rather than the cytoplasmic rate of P450R deadenylation or re-adenylation. Furthermore, nuclear export of P450R may be a slow process, as suggested by the time lag of ~4 h required for detection of the poly(A)-elongated P450R mRNA in the cytoplasm. It is unclear from the present studies, however, whether the rate of P450R RNA nuclear export is affected by thyroid hormone treatment. The T3-stimulated increase in P450R nuclear polyadenylation resulted in a discrete lengthening of the P450R mRNA's poly(A) tail, from ~100 A in the basal state to ~205 A in the T3-induced state. Little is known about the effects of thyroid hormone on nuclear polyadenylation activity, other than an early report indicating that thyroxine increases neuronal nuclear poly(A) polymerase activity in hypothyroid rats (Lindholm, 1984).

The T3-enhanced polyadenylation of P450R RNA was not sustained, insofar as the poly(A) tail length of nuclear P450R RNA returned to its basal length of ~100 A by 12 h after T3 treatment. Likewise, the poly(A) tail length of cytoplasmic P450R RNA returned to its basal length at 16 h. This time course is clearly distinct from the accumulation of cytoplasmic P450R mRNA, which is maximally elevated 16 to 24 h after T3 injection (Li et al., 2001). One interpretation of these findings is that P450R mRNA poly(A) tail lengthening is associated with the initial burst of T3-stimulated *P450R* transcription but is not sustained [compare maximal induction of nuclear P450R precursor RNA 12 h after T3 administration (Li et al., 2001)]. A mechanistic link between T3-induced *P450R* transcription and T3-stimulated nuclear P450R RNA polyadenylation is suggested by the fact that both processes were blocked by actinomycin D (Fig. 6), even

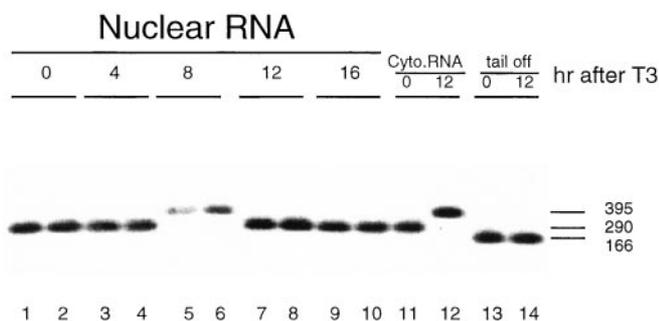


Fig. 5. T3 stimulates nuclear polyadenylation of P450R RNA. RNase H digestion was carried out as described in Fig. 4. Included in the analysis were nuclear RNA samples prepared from rat livers stimulated with T3 for 0 to 16 h (lanes 1–10) and total cytoplasmic RNA prepared from the same set of livers (0- and 12-h T3 samples, lanes 11–14). The RNase H digestion probe was ON-338 (lanes 1–12) or ON-338 + ON-293 (lanes 13 and 14) and the Northern blot hybridization probe was ON-329 (compare Fig. 1).

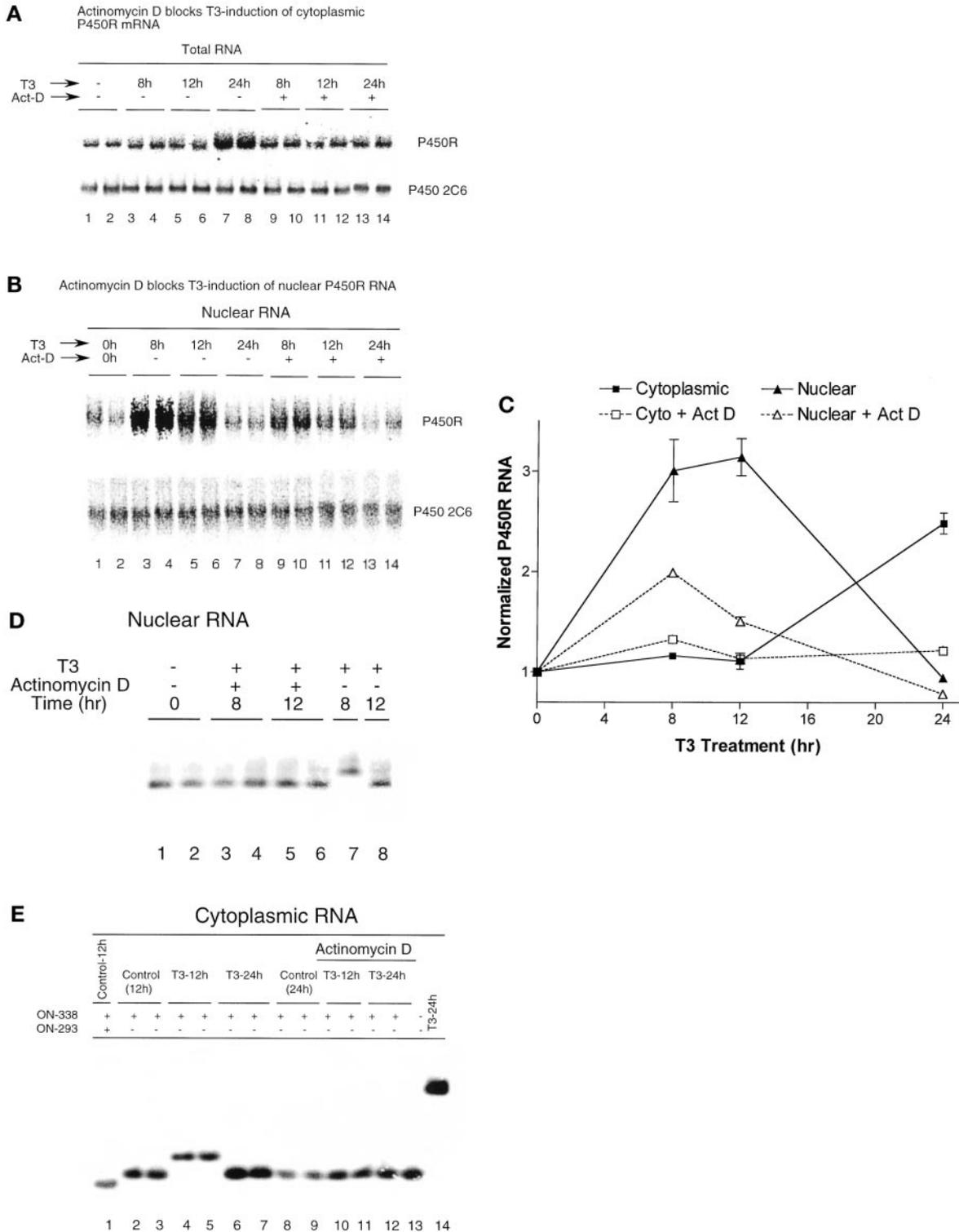


Fig. 6. Actinomycin D blocks T3-stimulated increase in P450R mRNA level (A–C) and the increase in P450R mRNA poly(A) tail length (D and E). Rats were killed 12 or 24 h after actinomycin D injection and livers were removed for isolation of RNA. Cytoplasmic (A) and nuclear (B) RNA samples were analyzed on Northern blots in comparison with P450 2C6 RNA, which is not T3-inducible and serves as a control. Note that the time course for cytoplasmic P450R induction seen in A (induction not detected until after 12 h) is somewhat delayed compared with that seen in the separate animal experiment shown in Fig. 1, where partial induction was already apparent by 12 h. C, PhosphorImaging quantitation of cytoplasmic and nuclear of P450R RNA levels normalized to P450 2C6 RNA levels, based on the data shown in A and B. Data graphed are mean values \pm half-range ($n = 2$ rats/data point). Actinomycin D (Act D) inhibited the induction of nuclear P450R RNA by 56% at 8 h and by 77% at 12 h. D and E, RNase H analysis of cytoplasmic (D) and nuclear liver P450R RNA (E) isolated from control or T3-treated rats (8-, 12-, or 24-h T3 treatment, as marked), without or with actinomycin D treatment. Actinomycin D blocks the T3-stimulated increase in poly(A) tail length seen in the nuclear RNA fraction at 8 h (D, lanes 3 and 4 versus lane 7) and in the cytoplasm at 12 h (E, lanes 10 and 11 versus lanes 4 and 5). E, lane 1 shows the 166-nt 3' fragment released from 12-h T3-induced P450R RNA upon RNase H digestion with ON-338 and ON-293 in combination. Lane 14 is a 24-h T3-induced RNA control sample without RNase H treatment.

though actinomycin D is not an inhibitor of the nuclear poly(A) polymerase per se. The inhibitory effect of actinomycin D on T3-induced P450R RNA polyadenylation may reflect a need for ongoing P450R transcription for the enhanced nuclear polyadenylation to be manifest or, alternatively, may reflect a requirement for a T3-induced gene product to increase P450R nuclear polyadenylation activity. Further study is needed to clarify this question.

Poly(A) tail length is an important regulatory feature in eukaryotic mRNAs, with cytoplasmic mRNA stability and translational activity often increased in mRNAs having a longer poly(A) tail (Ross, 1995). In the case of P450R, however, the increased polyadenylation of the cytoplasmic RNA seen at 12 h does not seem to be associated with a burst of translational activity, as suggested by data from an earlier study, where P450R protein and activity levels were monitored over the same time course used in the present hyperthyroid rat liver model (Ram and Waxman, 1992). Investigation of the effects of T3 on hepatic P450R RNA stability revealed a substantial (>6-fold) destabilization of this mRNA in T3-treated rat liver. This destabilization was observed 24 h after T3 treatment, when cytoplasmic P450R mRNA levels were maximally induced ($t_{1/2}$ of P450R mRNA ≥ 16 h in euthyroid liver compared with $t_{1/2} \sim 2.5$ h in hyperthyroid liver). Nuclear P450R RNA also exhibited a comparatively

short half-life in T3-induced liver ($t_{1/2} \sim 5$ h, determined 12 h after T3 treatment, when liver nuclear P450R RNA is maximal), although it could not be established whether this reflects destabilization compared with the corresponding uninduced nuclear P450R RNA, which was expressed at too low a level for reliable half-life measurement. The decrease in cytoplasmic P450R mRNA stability in T3-treated liver is, however, unrelated to the stimulatory effect of T3 on P450R polyadenylation, which as noted above, is transient and was no longer detectable 16 h after T3 administration. Accordingly, the mechanism whereby T3 destabilizes cytoplasmic P450R mRNA at 24 h is unclear. However, in view of the absence of P450R RNA structural alterations, this destabilization seems likely to involve a *trans*-acting factor controlling mRNA decay (Wilson and Brewer, 1999) (e.g., a T3-regulated P450R RNA-binding protein). The destabilization of P450R RNA by T3 is likely to contribute to the dramatic decline in the induced P450R message that is seen in hyperthyroid rats, both in the nucleus (by 24 h after T3 treatment) and the cytoplasm (36 h after T3) (Ram and Waxman, 1992; Li et al., 2001). T3 effects on mRNA turnover described previously include both mRNA stabilization [pituitary growth hormone and liver HMG-CoA reductase RNAs (Murphy et al., 1992; Simonet and Ness, 1988)] and mRNA destabilization [epidermal growth factor and pituitary thyrotropin- β RNAs; (Kesavan et al., 1991; Krane et al., 1991)].

The functional significance of the two post-transcriptional effects of T3 on P450R RNA described in this study is uncertain. As noted above, the transient increase in cytoplasmic P450R poly(A) tail length is not associated with any apparent increase in P450R mRNA translatability, as suggested by the absence of a T3-stimulated increase in P450R protein levels at this point in time (Ram and Waxman, 1992). One consequence of the subsequent destabilization of cytoplasmic P450R mRNA may be to limit the extent to which T3 induces liver P450R activity, perhaps as a mechanism to moderate P450R-dependent redox metabolism and oxidative stress. Additional post-transcriptional effects of T3 on P450R can be anticipated, as indicated by the apparent reduced translational activity of P450R mRNA observed in hyperthyroid rat liver (Ram and Waxman, 1992).

Acknowledgments

We thank Dr. Huanchen Li for preliminary RNase H digestion experiments.

References

- Bligh HFJ, Bartoszczek A, Robson CN, Hickson ID, Kasper CB, Beggs JD, and Wolf CR (1990) Activation of mitomycin C by NADPH: cytochrome P450 reductase. *Cancer Res* **50**:7789–7792.
- Brown T (1997) Analysis of RNA by Northern and slot blot hybridization, in *Current Protocols in Molecular Biology* (Ausbel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K eds) pp 4.9.1–4.9.8, John Wiley & Sons, Inc., New York.
- Cawley GF, Batie CJ, and Backes WL (1995) Substrate-dependent competition of different P450 isozymes for limiting NADPH-cytochrome P450 reductase. *Biochemistry* **34**:1244–1247.
- Chen Q and Cederbaum AI (1997) Menadione cytotoxicity to Hep G2 cells and protection by activation of nuclear factor- κ B. *Mol Pharmacol* **52**:648–657.
- Chesis PL, Levin DE, Smith MT, Ernster L, and Ames BN (1984) Mutagenicity of quinones: pathways of metabolic activation and detoxification. *Proc Natl Acad Sci USA* **81**:1696–1700.
- Chkheidze AN, Lyakhov DL, Makeyev AV, Morales J, Kong J, and Liebhaber SA (1999) Assembly of the alpha-globin mRNA stability complex reflects binary interaction between the pyrimidine-rich 3' untranslated region determinant and poly(C) binding protein alphaCP. *Mol Cell Biol* **19**:4572–4581.
- Choi J, Liu RM, and Forman HJ (1997) Adaptation to oxidative stress: quinone-mediated protection of signaling in rat lung epithelial L2 cells. *Biochem Pharmacol* **53**:987–993.

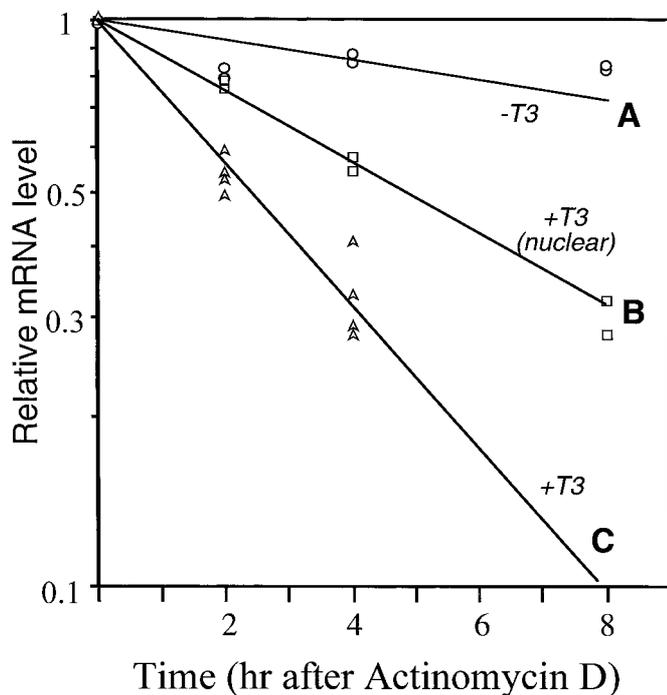


Fig. 7. Decrease in P450R mRNA half-life in T3-treated rat liver. Cytoplasmic and nuclear liver RNA was extracted from untreated or T3-induced rats at the indicated times after further transcription of P450R RNA was blocked by injection of actinomycin D. Samples were analyzed for P450R RNA by Northern blotting, and hybridization signals were quantitated and graphed on a semilogarithmic plot. The P450R mRNA level at the time of actinomycin D injection was assigned an arbitrary value of 1 for each treatment group. Curve A, cytoplasmic P450R RNA in the absence of T3 treatment; curve B, T3-induced nuclear P450R RNA; curve C, T3-induced cytoplasmic P450R RNA. Each data point corresponds to an individual rat liver RNA sample (i.e., $n = 2-4$ rats per time point for each experiment). For the experiments shown in curves A and C, actinomycin D ($t = 0$ h) was injected 24 h after T3 injection. For experiment B, actinomycin D ($t = 0$ h) was injected 12 h after T3 injection, when nuclear P450R RNA is maximally elevated.

- Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**:156–159.
- Connor MK, Takahashi M, and Hood DA (1996) Tissue-specific stability of nuclear- and mitochondrially encoded mRNAs. *Arch Biochem Biophys* **333**:103–108.
- Gonzalez FJ and Kasper CB (1981) Sequential translocation of two phenobarbital-induced polysomal messenger ribonucleic acids from the nuclear envelope to the endoplasmic reticulum. *Biochemistry* **20**:2292–2298.
- Kaminsky LS and Guengerich FP (1985) Cytochrome P450 isozyme/isozyme functional interactions and NADPH-cytochrome P450 reductase concentrations as factors in microsomal metabolism of warfarin. *Eur J Biochem* **149**:479–489.
- Kesavan P, Mukhopadhyay S, Murphy S, Rengaraju M, Lazar MA, and Das M (1991) Thyroid hormone decreases the expression of epidermal growth factor receptor. *J Biol Chem* **266**:10282–10286.
- Kingston RE, Chomczynski P, and Sacchi N (1996) CsCl purification of RNA from tissue, in *Current Protocols in Molecular Biology* (Ausbel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K eds) pp 4.2.5–4.2.9, John Wiley & Sons, Inc., New York.
- Krane IM, Spindel ER, and Chin WW (1991) Thyroid hormone decreases the stability and the poly(A) tract length of rat thyrotropin beta-subunit messenger RNA. *Mol Endocrinol* **5**:469–475.
- Landriscina C, Petragallo V, Morini P, and Marcotrigiano GO (1988) Lipid peroxidation in rat liver microsomes. I. Stimulation of the NADPH-cytochrome P-450 reductase-dependent process in hyperthyroid state. *Biochem Int* **17**:385–393.
- Li HC, Liu D, and Waxman DJ (2001) Transcriptional induction of hepatic NADPH: cytochrome P450 oxidoreductase by thyroid hormone. *Mol Pharmacol* **59**:987–995.
- Lindholm DB (1984) Thyroid hormone regulation of poly(adenylate) polymerase activities in neuronal nuclei of developing rat brain cortex. *Biochem Biophys Res Commun* **125**:931–937.
- Manley JL (1995) Messenger RNA polyadenylation: a universal modification. *Proc Natl Acad Sci USA* **92**:1800–1801.
- Mano H, Mori R, Ozawa T, Takeyama K, Yoshizawa Y, Kojima R, Arai Y, Masushige S, and Kato S (1994) Positive and negative regulation of retinoid X receptor gene expression by thyroid hormone in the rat. Transcriptional and post-transcriptional controls by thyroid hormone. *J Biol Chem* **269**:1591–1594.
- Minvielle-Sebastia L and Keller W (1999) mRNA polyadenylation and its coupling to other RNA processing reactions and to transcription. *Curr Opin Cell Biol* **11**:352–357.
- Murphy D, Pardy K, Seah V, and Carter D (1992) Posttranscriptional regulation of rat growth hormone gene expression: increased message stability and nuclear polyadenylation accompany thyroid hormone depletion. *Mol Cell Biol* **12**:2624–2632.
- O'Leary KA, Beck TW, and Kasper CB (1994) NADPH cytochrome P450 oxidoreductase gene: identification and characterization of the promoter region. *Arch Biochem Biophys* **310**:452–459.
- O'Leary KA, Li HC, Ram PA, McQuiddy P, Waxman DJ, and Kasper CB (1997) Thyroid regulation of NADPH cytochrome P450 oxidoreductase: identification of a thyroid responsive element in the 5'-flank of the oxidoreductase gene. *Mol Pharmacol* **52**:46–53.
- O'Leary KA, Shen AL, and Kasper CP (2000) The mouse NADPH-cytochrome P450 oxidoreductase gene: identification of multiple promoters (Abstract). *FASEB J* **14**:A1333.
- Porter TD (1991) An unusual yet strongly conserved flavoprotein reductase in bacteria and mammals. *Trend Biochem Sci* **16**:154–158.
- Porter TD, Beck TW, and Kasper CB (1990) NADPH-cytochrome P450 oxidoreductase gene organization correlates with structural domains of the protein. *Biochemistry* **29**:9814–9818.
- Ram PA and Waxman DJ (1992) Thyroid hormone stimulation of NADPH P450 reductase expression in liver and extrahepatic tissues. Regulation by multiple mechanisms. *J Biol Chem* **267**:3294–3301.
- Ross J (1995) mRNA stability in mammalian cells. *Microbiol Rev* **59**:423–450.
- Sawamura AO, Aoyama T, Tamakoshi K, Mizuno K, Suganuma N, Kikkawa F, and Tomoda Y (1996) Transfection of human cytochrome P-450 reductase cDNA and its effect on the sensitivity to toxins. *Oncology* **53**:406–411.
- Simonet WS and Ness GC (1988) Transcriptional and posttranscriptional regulation of rat hepatic 3-hydroxy-3-methylglutaryl-coenzyme A reductase by thyroid hormones. *J Biol Chem* **263**:12448–12453.
- Sindhvani R, Ismail-Beigi F, and Leinwand LA (1994) Post-transcriptional regulation of rat alpha cardiac myosin heavy chain gene expression. *J Biol Chem* **269**:3272–3276.
- Stoeckle MY and Guan L (1993) High-resolution analysis of gro alpha mRNA poly(A) shortening: regulation by interleukin-1 beta. *Nucleic Acids Res* **21**:1613–1617.
- Strobel HW, Hodgson AV, and Shen S (1995) NADPH cytochrome P450 reductase and its structural and functional domains, in *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano PR ed) pp 225–244, Plenum Press, New York.
- Sundseth SS, Alberta JA, and Waxman DJ (1992) Sex-specific, growth hormone-regulated transcription of the cytochrome P450 2C11 and 2C12 genes. *J Biol Chem* **267**:3907–3914.
- Waxman DJ (1991) Rat hepatic P450IIA and P450IIC subfamily expression using catalytic, immunochemical, and molecular probes. *Methods Enzymol* **206**:249–267.
- Waxman DJ, Morrissey JJ, and LeBlanc GA (1989) Hypophysectomy differentially alters P-450 protein levels and enzyme activities in rat liver: pituitary control of hepatic NADPH cytochrome P-450 reductase. *Mol Pharmacol* **35**:519–525.
- Williams DW, Sensel M, McTigue M, and Binder R (1993) Hormonal and developmental regulation of mRNA turnover, in *Control of Messenger RNA Stability* (Belasco JG and Brawerman G eds) pp 161–197, Academic Press, Inc., San Diego, CA.
- Wilson GM and Brewer G (1999) The search for *trans*-acting factors controlling messenger RNA decay. *Prog Nucleic Acid Res Mol Biol* **62**:257–291.

Address correspondence to: David J. Waxman, Ph.D., Department of Biology, Boston University, 5 Cummington Street, Boston, MA. E-mail: djw@bu.edu
