Negative Regulation of the Gene for the Preprothyrotropin-releasing Hormone from the Mouse by Thyroid Hormone Requires Additional Factors in Conjunction with Thyroid Hormone Receptors*

(Received for publication, June 5, 1996, and in revised form, July 30, 1996)

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To gain additional insights into the negative gene regulatory action by triiodothyronine (T₃), we isolated a 2-kilobase pair 5'-flanking region of the mouse preprothyrotropin-releasing hormone (ppTRH) gene and characterized the DNA elements mediating inhibitory regulation by T₃ in the promoter region. In GH₃ cells, the expression of the 2-kilobase pair mouse ppTRH 5'-flanking region fused to the luciferase reporter gene occurred by transfection and was significantly suppressed by T₃. In contrast, T₃ suppression was not observed in T₃ receptor (T₃R)-deficient CV-1 cells, suggesting that T₃Rs were required for the negative regulation. Cotransfected mouse T₃R α₁, β₁, and β₂ possessed indistinguishable potency for the negative regulation. Deletion analysis localized the element mediating the negative regulation to the region between −83 and +46, and the sequence downstream of the transcription start site (TSS) between +12 and +46 was found to be essential for the inhibitory regulation. In mobility shift assays, only T₃R monomers bound to the element containing a T₃ response element half-site at −57. No apparent T₃R binding was observed to the element downstream of TSS. Neither the T₃ response element half-site nor the element downstream of the TSS confer T₃ suppression individually in heterologous promoters. These results indicate that the negative regulation of murine ppTRH gene by T₃ might be mediated by the cooperation of T₃R monomers with unknown factor(s) interacting with the element downstream of the TSS.

Thyrotropin-releasing hormone (TRH) is the hypothalamic tripeptide stimulating thyrotropin (TSH) synthesis and secretion in the anterior pituitary gland (1). TRH is derived from a large precursor protein, preproTRH (ppTRH), by posttranslational processing and enzymatic modification (2). The expression of the ppTRH gene in the paracellular subdivision of the paraventricular nucleus in the hypothalamus is negatively regulated by thyroid hormones (3, 4). Recently, we and others demonstrated that the promoter activities of human and rat ppTRH genes were directly suppressed by triiodothyronine (T₃) (5–7). These results indicated that the ppTRH gene belongs to a family of T₃-responsive genes, including the α and β subunits of TSH genes, β-myosin heavy chain gene, and the epidermal growth factor receptor gene, whose expression are negatively regulated by T₃ at the level of gene transcription (8–15).

Thyroid hormone receptor (T₃R) is the ligand-dependent transcriptional factor that activates or inhibits gene transcription basically by binding to the specific cis-acting DNA elements, so-called thyroid hormone response elements (T₃REs), located in the 5'-flanking regions of T₃R-responsive genes (16, 17). To date, the negative T₃REs (nT₃REs) in several gene promoters have been characterized (8–15). In the TSHβ subunit and glycoprotein α subunit genes, the nT₃REs reside near the TATA box and contain a single hexamer sequence matching perfectly or loosely to the consensus core half-site motifs for T₃RE (8–13). It has therefore been speculated that T₃Rs binding to the inhibitory T₃REs mediate transcriptional suppression by steric interference with the transcription initiation machinery. In contrast, the nT₃RE in epidermal growth factor receptor gene is localized between −112 and −76, and it contains a single TRE half-site-like motif (GCGGACT) which overlaps with the Sp1 binding site (15). The nT₃RE weakly binds T₃R homodimers, and an addition of nuclear extracts from HeLa cells augments T₃R binding with formation of T₃R/T₃R auxillary protein heterodimers in the EMSA (18). It has been recently demonstrated that the hormone-responsive element of the Rous sarcoma virus long terminal repeat consisting of a novel inverted palindrome with a 6-bp spacer (TGCCATT-AAGGAAGGA) is activated by unliganded TRα1, but not by TRβ1, and the effect is reversed by an addition of T₃ (19). The T₃RE in the Rous sarcoma virus promoter binds TRα homodimers and RXR/TRα heterodimers (19). These results indicate that T₃Rs are capable of exerting inhibitory regulation of gene transcription through structurally divergent nT₃REs, which seems to be analogous to transcriptional activation by T₃ through a variety of positive TREs, such as the palindromic T₃RE (PAL), the direct repeat of the half-sites with a 4-base pair (bp) spacer (DR4), and the inverted palindrome with a 6-bp spacer (IP-6) (20). However, the detailed mechanism by which T₃ regulates gene transcription positively or negatively remains to be determined.

A detailed analysis of the human ppTRH gene promoter recently demonstrated that both the binding of T₃Rs as heterodimers with the 9-cis-retinoic acid receptors (RXR) to the element that contains a single TRE half-site located 60 bp upstream of the TSS and the binding of T₃R monomers to the...
two TRE half-sites positioning downstream of the TSS were required to exhibit full-inhibition of the human ppTRH gene promoter by T₃ (7). Moreover, one of the functional T₃R isofoms, TRβ₁, preferentially mediates the negative regulation (7). These results provided direct evidence of the involvement of RXR and T₃R isofom specificity in the negative regulation of the human ppTRH gene promoter by T₃.

In order to gain further insight into the negative gene regulatory action by T₃, we cloned a 2-kb fragment of the 5'-flanking region of the mouse ppTRH gene and sought to identify the cis-acting elements necessary for the negative regulation by T₃. The present results indicate that the mechanism involved in the inhibitory regulation of the mouse ppTRH gene promoter by T₃ is distinct from that of the human ppTRH gene.

MATERIALS AND METHODS

Cloning of the 5'-Flanking Region of the Mouse PreproTRH Gene—A mouse T₃R cell genomic library (kindly provided by T. Aizawa (21)) was screened by the standard method described elsewhere using a 2-flanked mouse ppTRH cDNA previously isolated in this laboratory (22). The isolated clone was mapped by restriction endonuclease digestions in combination with Southern blot analyses. Two overlapping DNA fragments containing the promoter region and a part of the 5'-translational region (HS2) and 5'-untranslated region, the first exon and a part of the first intron (SB2.0) excised with HindIII and Sall or SalI and BamHI digestion, respectively, from the isolated clone were subcloned into pGEM 4Z (Promega). The nucleotide sequence for both strands of these clones were determined by the dyeide chain termination method (23) using Sequenase Version 2 (U.S. Biochemical Corp.). Nucleotide sequences were analyzed using a computer program, GENETYX (Software Development Co., Ltd.).

Oligonucleotides—Identical oligonucleotides with HindIII linkers at 5' and 3' ends were used for the construction of heterologous promoters and EMISA. The nucleotide sequence of the upper strands of these oligonucleotides were as follows (underlines indicate TRE half-site motifs, and lowercase letters indicate HindIII linker sequences): PAL, 5'-AGCTATCAGGTCATGACCTGCGA-3'; TRE1/2, 5'-AGCTTGACCTGGGA-3'; Site 5&6 5'-AGCTTCCCTCGACCTGACCTACA-3'; TRE1/2', 5'-AGCTTCCCTCGACCTGACCTACA -3'; Site 5&6', 5'-AGCTTGACCTGGGA-3'; PAL6, 5'-AGCTTGACCTGGGA-3'.

Phusion Construction—Using HS2.0 and SB2.0 fragments, a series of plasmids containing various lengths of the promoter and 5'-untranslated regions of the mouse ppTRH gene were constructed in pGEM 4Z or 11ZI (Promega) by appropriate restriction enzyme digestions or amplification by polymerase chain reactions. The DNA fragments excised from these pGEM vectors were subsequently inserted into the multiple cloning sites of a reporter plasmid, pκLuc, which contains the firefly luciferase cDNA as a reporter (kindly provided by W. M. Wood (24)). The heterologous promoters, IP-5', TRE1/2', Site 5&6', and PAL6-TKLuc plasmids were constructed by ligation of the double-stranded oligonucleotides described above into the unique HindIII site of the pκT09Luc, which possesses the minimal promoter of Herpes Simplex virus thymidine kinase gene (25). The orientation and number of inserts in these reporter constructs were verified by DNA sequencing. Heterologous constructs that possessed a single copy of an individual oligonucleotide in correct orientation were further used for transfection experiments.

Cell Culture, Transient Transfection, and Luciferase Assay—GH₃C₁, GH₃, and CV1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml) (Life Technologies, Inc.), and amphotericin B (0.25 µg/ml) (Sigma). Cells were split 24 h before transfection into 60-mm tissue culture dishes in subconfluence. Transient transfection was performed in triplicate plates in all experiments by the calcium phosphate precipitation method using Cellfect (Pharmacia Biotech Inc.) with 3 µg of reporter constructs. For cotransfection experiments, 150 ng of expression vectors for the mouse T₃R(1), β₁, and β₂ driven by Rous sarcoma virus promoter (pBSVmTrβ₁, β₁, and β₂ kindly provided by W. M. Wood) (26, 27) were transfected with TRH reporter constructs. Glycerol shock was performed 16 h after transfection for 2 min except for CV-1 cells, and the cell culture medium was changed to Dulbecco’s modified Eagle’s medium without phenol-red supplemented with 10% fetal bovine serum treated with AG1-X8 resin (Bio-Rad) and activated charcoal (Sigma) to remove thyroid and steroid hormones. Cells were incubated for an additional 72 h with or without T₃ (10-10 to all-trans retinoic acid (1 µM)). Luciferase assays were carried out as described previously (6, 28). In brief, cells were rinsed twice with 5 ml of phosphate-buffered saline and harvested with 400 µl of a buffer containing 1% Triton X-100, 25 µM glycylglycine, 15 mM MgSO₄, 4 mM EDTA, and 1 mM dithiothreitol. Luciferase activities were quantitated with 300 µl of cell extracts by integration of light readings over 10 s on a standard luminometer (Chiba Corning) following injection of 0.1 ml of an assay buffer containing 0.2 mM luciferin (Wako), 15 mM K₂HPO₄, 2 mM ATP, and 1 mM dithiothreitol. Protein concentrations were determined by the Bradford method using bovine serum albumin (Sigma) as a standard (29). Luciferase activities were normalized by protein concentration and expressed as light units/µg protein.

Electrophoretic Gel Mobility Shift Assay—The double-stranded oligonucleotides described above were radiolabeled by fill-in reaction of the 5' overhangs with [α-32P]dCTP (3000 Ci/mmol, Du Pont NEN) using a Klenow fragment of DNA polymerase I (Takara) and were purified using Sephadex G-25 columns (Boehringer Mannheim). Human TRβ₁ and RXRα were synthesized from non-linearized pEa101 and pBS-RXRα (kindly provided by R. M. Evans) (30, 31), respectively by in vitro translation using TNT-coupled rabbit reticulocyte lysates (Promega) according to the supplier’s manual. Synthetic proteins in expected molecular weights was confirmed by labeling of in vitro translated products with [35S]methionine and cysteine (ExpreS²IS²S, DuPont NEN), followed by SDS-polyacrylamide gel electrophoresis analysis (data not shown). Binding reactions were performed for 20 min at room temperature in a total volume of 20 µl with 1 µg of poly(dI-dC) (Pharmacia), 20 mM HEPES (pH 7.6), 50 mM KCl, 20% glycerol, 1 mM dithiothreitol, 100,000 cpm of purified probes, and 4 µl of in vitro synthesized reaction mixtures. For competitive experiments, 200-fold molar excess of cold oligonucleotides were included unless otherwise indicated. For supershift experiments, 2 µl of the specific antibody raised against a synthetic peptide corresponding to the N-terminal region of the TRβ1 (amino acid 62–82) was incubated with binding reaction mixtures for an additional 20 min at 4°C (Affinity BioRegent). Binding reaction mixtures were loaded on 5% nondenaturing polyacrylamide gels and were separated in 0.5 × TBE buffer (20 × TBE: 1× Tris, 1× boric acid, and 20 µM EDTA-Na₂) at 250 V for 80 min at room temperature. Autoradiography was carried out for 16–48 h with an intensifying screen at ~80°C.

RESULTS

Characterization of the 5'-Flanking Region of the Mouse PreproTRH Gene—By screening of a mouse genomic library with a mouse hypothalamic ppTRH cDNA probe, we isolated an approximately 10-kb mouse ppTRH gene possessing a 6-kb 5'-flanking region, three exons interrupted by two introns, and an entire 3'-untranslated region. To study the regulation of the mouse ppTRH gene promoter by T₃, we further characterized a 2-kb 5'-flanking region. To date, the nucleotide sequences of 5'-flanking regions of the rat and human ppTRH genes have been reported up to ~494 and ~243 bp, respectively, from the position of TSS (32, 33). We have therefore sequenced the longest 5'-flanking region of mammalian ppTRH genes (Fig. 1). Sequence analyses revealed that a putative TATA box (TATAA) was located in the position similar to that of the rat and human ppTRH gene promoters. A putative Sp1 binding site (GGGCGG) found in the human and rat TRH gene promoters was also conserved in the mouse gene 117 bp upstream of the TSS. No CCAAT box was found, as is the case with other species. Two octameric sequences homologous to the consensus binding site (ATTGGCAT) of POU homeodomain proteins (34) were found at positions ~1174 (ACTTGGCAT) and ~635 (ATTGCGCT). The consensus half-site sequences for the T₃RE (AGGTCA or TGACCC) were found at three separate positions in different arrangements. An inverted palindrome with a 5-bp binding site (ATTGCGCT) was found at position ~1876, which resembled the alignment of the positive T₃RE identified in chick lysozyme gene promoter.

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* M. Yamada, T. Sato, T. Iwasaki, and M. Mori, unpublished observation.
(TGACCCCAGCTGAGGTCA) (35). An octameric T₃RE (TGAC-CTCA, designated as TRE1/2) was found at position −257. Moreover, a palindrome with a 6-bp spacer (AGGTAATGCCTCTGACCT, designated as PAL6) was identified at position 1102, which overlapped with the first exon-intron junction and resembled the alignment of T3RE identified in herpes simplex virus thymidine kinase promoter (AGGTGACGCGTGTGGC) (36). Two TRE half-sites positioned downstream of the TSS in the human ppTRH gene (GGGTCC and TGACCT) (7) were not conserved in the mouse gene. A putative half-site sequence for the glucocorticoid response element (TGTTCT) found in the rat and human ppTRH gene promoters was also conserved in the mouse gene at position 2208. The overall sequence homologies to the 5'-flanking regions of rat and human ppTRH gene were 80.9% and 50.8%, respectively.

**Negative Regulation of Mouse PreproTRH Gene Promoter by T₃**

We first examined expression of the reporter construct, in which the 21893/1127 fragment (including the promoter, 5'-untranslated regions, and a part of the first intron of the mouse ppTRH gene) was fused to the firefly luciferase cDNA, using transient transfection into GH3 and GH4C1 cells. These pituitary tumor cell lines have been known to express functional T3Rs endogenously and are utilized extensively to study negative regulation of promoter activities by T3 (10–12). When chimeric constructs were transfected into two cell lines in parallel, approximately 10 times higher luciferase activities were observed in GH4C1 cells than in GH3 cells (data not shown). We therefore used GH4C1 cells for further experiments. As shown in Fig. 2, 10 nM T3 suppressed luciferase activity of the longest construct (21893/1127) about 2.2-fold. In contrast, T₃ did not influence TK promoter activities significantly (Fig. 6). More-
Deletion of the sequence between known to be deficient for endogenous T3Rs. PALTK-Luc, in formed cotransfection experiments using CV-1 cells, which are whether transcriptional inhibition is mediated by T3Rs and 1 activities of PALTK-Luc or TRH-Luc (constructs. Cotransfected mouse TR 
stream of the TK promoter, was used as a positive control. As that the element between 12 and +127 was necessary for the negative regulation. Deletion of the PAL6 motif (−254/+87) did not significantly reduce T3 suppression compared with the −254/+127 construct, indicating that PAL6 was not involved in the inhibitory regulation. Finally, the shortest construct (−83/+46) was significantly suppressed by T3. These data imply that the promoter-proximal element between −83 and +46 contains the DNA element necessary for the inhibitory regulation by T3 in the mouse ppTRH gene promoter.

**T3Rα1, β1, and β2 Possess Equal Potency for the Inhibitory Effect by T3 on the Mouse ppTRH Gene Promoter**—To study whether transcriptional inhibition is mediated by T3Rs and whether T3R isomorphism-specificity is involved in the negative regulation of the mouse ppTRH gene promoter by T3, we performed cotransfection experiments using CV-1 cells, which are known to be deficient for endogenous T3Rs. PALTK-Luc, in which two copies of the palindromic T3RE were ligated upstream of the TK promoter, was used as a positive control. As shown in Fig. 3A, 10 nM T3 did not influence the basal promoter activities of PALTK-Luc or TRH-Luc (−1893/+127) reporter constructs. Cotransfected mouse TRβ1 repressed basal promoter activities of PALTK-Luc approximately 3-fold without its ligand, and the addition of T3 activated the transcription about 30-fold. In contrast, unliganded TRβ1 stimulated the basal expression of TRH-Luc reporter approximately 2-fold, and 10 nM T3 reversed this activation. These ligand-independent and -dependent effects by T3Rs were consistent with those observed by us with human ppTRH promoter in a neuroblastoma cell line (6). These results suggest that cotransfected T3Rs play pivotal roles in mediating stimulatory or inhibitory effects of T3 on the ppTRH gene transcription in CV-1 cells. However, in contrast to the human ppTRH gene regulation (7), no significant difference was observed in T3-mediated inhibitory potency among three functional isoforms of the mouse T3Rα1, β1, and β2 (Fig. 3B).

**Electrophoretic Mobility Shift Assays**—To examine whether T3Rs bind to the putative T3REs found in the 5’-flanking region of the mouse ppTRH gene, EMSA was carried out using in vitro translated TRβ1 and radiolabeled oligonucleotides containing IP-5 (−1893/−1862), TRE1/2 (−73/−47), or PAL6 motifs (+97/+123). It has been reported that T3R monomers bound to two TRE half-sites located downstream of the TSS in the human ppTRH gene promoter (7). Although these TRE half-sites were not conserved in the mouse gene, we examined whether T3Rs bind to the element of the mouse gene (here designated as Site 5866, +14/+47). An oligonucleotide containing the idealized palindromic T3RE (PAL) was used as a positive control for EMSA. Nonspecific binding was assessed by incubation with unprogrammed reticulocyte lysates. As shown in Fig. 4A, a faint band corresponding to the TR-homodimer formation was observed on PAL, and this homodimer-DNA complex was diminished by addition of 200-fold molar excess of unlabeled oligonucleotides. Moreover, the band was supershifted by incubation with a specific antibody for human TRβ1, confirming the
binding specificity. The apparent homodimer formation was observed also upon IP-5. In contrast, a faster migrating band representing T3R monomer-DNA complex was observed on TRE1/2 oligonucleotide, and this complex was also super-shifted. As anticipated, no significant T3R binding was detected on Site 5&6 sequence as well as on PAL6 (data not shown). We next examined whether RXR/T3R heterodimers bind to these oligonucleotides. As shown in Fig. 4B, strong heterodimer formation was observed on PAL. A heterodimer band with a slightly faster mobility than that formed on PAL was also detected on IP-5 with an intensity similar to that of homodimer band (Fig. 4B). Neither heterodimer formation nor RXR binding was observed on TRE1/2, Site 5&6, or PAL6 (data not shown). The presence of 100 nM T3 dissociated T3R homodimers, but not heterodimers, from the IP-5 (Fig. 4C). To further confirm that T3Rs bind to the TRE half-site at 257 and not to Site 5&6, we performed competition experiments to find whether RXR/T3R heterodimer formation on the PAL is inhibited by two overlapping fragments containing the TRE half-site (TRE1/2 and TRE1/2') and Site 5&6. As shown in Fig. 5B, addition of TRE1/2 and TRE1/2' efficiently inhibited heterodimer binding to PAL in the manner similarly to unlabeled PAL (Fig. 5A), indicating that the overlapping 15-bp sequence in the two competitors (CCGGCGAACCTACA) possessed a T3R-binding site. In contrast, Site 5&6 did not inhibit heterodimer formation, further confirming that T3Rs could not bind to this element (Fig. 5A).

Neither the T3R Half-site nor the Downstream Element Functioned Individually as nT3REs in Heterologous Promoters—To examine whether putative T3R REs found in the mouse ppTRH 5'-flanking region independently confer transcriptional stimulation or inhibition by T3, heterologous promoters were constructed in which oligonucleotides carrying an IP-5, TRE1/2, Site 5&6, or PAL6 motif were fused in front of the TK promoter, and were transiently transfectected into GH3 cells. As shown in Fig. 6, 10 nM T3 stimulated luciferase activities of the PALT K about 5-fold. In contrast, no significant stimulation by T3 was
mediates transcriptional activation by T3 in GH4C1 cells and been reported that the PAL6 motif in the viral TK promoter which overlapped with the first exon-intron junction. It has T3 suppression, presumably in a position-dependent manner. The element downstream of the TSS cooperatively mediate the heterologous constructs, suggesting that the TRE half-site and T3REs demonstrated that the spacing in IP response elements by the 3-to-5 rule (38). A recent detailed analysis of IP-type is well known to be critical to determine receptors specificity, as direct repeat response elements, the length of the spacer region inverted palindrome with a 5-bp spacer (IP-5) at (IP-2), the human TR ergodimers bound to IP-5 in the EMSA, this element did not function as T3REs in the heterologous construct. The TRE1/2, Site 5&6, or PAL6 were inserted upstream of the TK promoter. The heterologous constructs were transfected into GH4C1 cells with or without 10 nM T3. Data represent mean ± S.E. from three separate transfections.

observed in TK109-Luc. Although T3R monomers and heterodimers bound to IP-5 in the EMSA, this element did not function as T3REs in the heterologous construct. The TRE1/2 and Site 5&6 motifs, which were located in the sequence necessary for the negative regulation of the mouse ppTRH gene promoter by T3, did not function independently as nT3REs in heterologous constructs, suggesting that the TRE half-site and the element downstream of the TSS cooperatively mediate the T3 suppression, presumably in a position-dependent manner. Moreover, PAL6-TK-Luc did not show transcriptional activation or inhibition by T3.

**DISCUSSION**

In the present study, we characterized the 2-kb 5'-flanking region of the mouse ppTRH gene and localized its nT3RE in the promoter region. The 5'-flanking region of the mouse ppTRH gene contained T3RE-like motifs, which resembled previously characterized positive T3REs, at two separate positions, an inverted palindrome with a 5-bp spacer (IP-5) at −1876 and a palindromic TRE with a 6-bp spacer (PAL6) at +102. The natural inverted palindromic sequences with differential spacing have been found in the chicken embryonic myosin gene (IP-2), the human TRβ promoter (IP-5), the chicken lysozyme silencer element (IP-6), and myelin basic protein (IP-6) (37). Among these natural IP motifs, only the IP-6 in two chicken genes are able to mediate transcriptional activation by T3. In direct repeat response elements, the length of the spacer region is well known to be critical to determine receptor specificity, as by the 3-to-5 rule (38). A recent detailed analysis of IP-type T3REs demonstrated that the spacing in IP response elements is also important to determine the binding characteristics of T3R homodimers and RXR/T3R heterodimers by EMSA (37). IP-5 motif found in the mouse gene exclusively as monomers in the EMSA. These results suggest that the sequence between two half-sites and/or 5'- and 3'-flanking sequences of the half-sites might be crucial to mediate T3 action on the PAL6 motif.

In the transfection study with CV-1 cells, cotransfected T3Rs stimulated basal promoter activities of the mouse ppTRH gene without T3, and an addition of T3 repressed this basal stimulation, indicating that T3 Rs are required for the negative regulation of the mouse ppTRH gene promoter by T3. Deletion analyses revealed that the nT3RE of the mouse ppTRH gene was located in the promoter-proximal element between −83 to +46. The nucleotide sequence of the region upstream of the TATA box in the mouse gene was highly conserved when compared with those of rat and human ppTRH genes (Fig. 7). In this region, a perfectly matched TRE half-site (TGACCT) was conserved in all species, and the two flanking nucleotides 3' of this half-site were matched to the consensus sequence for the proposed octameric TRE half-site sequence, (T/C)(A/G)AG-

*Fig. 6. Effect of T3 on the heterologous reporter genes transfected into GH4C1 cells. Oligonucleotides containing PAL-, IP-5, TRE1/2, Site 5&6, or PAL6 were inserted upstream of the TK promoter. The heterologous constructs were transfected into GH4C1 cells with or without 10 nM T3. Data represent mean ± S.E. from three separate transfections.

**Fig. 7. Comparison of the nucleotide sequences of promoter-proximal elements of mouse, rat, and human ppTRH genes. Boxed letters indicate different nucleotides among three species. Capital letters indicate the sequence of the first exon. The T3 RE half-site sequences and TATA box are underlined.*
by T₃. Unexpectedly, heterologous constructs containing either the TRE1/2 or the downstream element placed in front of the TK promoter did not independently confer negative regulation by T₃. It has been reported that a 17-bp motif (GGCCAGTGCAAAATGAAG) located at the 3′ end of exon 1 of the rat TSH β gene containing a single copy of a hexamer TRE half-site with some degeneracy mediates T₃ inhibition in an orientation- and position-independent manner when fused to the TK/CAT reporter in GH₃ cells (40). The nThrRE of rat TSHβ bound in vitro synthesized T₃R monomers (40). In contrast, it has been demonstrated that T₃R monomers also bound to the octameric TRE half-site in the mouse ppTRH gene promoter was not taken together, suggest that the negative regulation of the gene is negatively regulated by T₃ (49). Thus, the possibility has been raised that this T₃R isoform plays a pivotal role in negative feedback action by T₃ in the hypothalamo-pituitary-thyroid axis. In the present study, we did not observe any significant functional difference among the three mouse T₃R isoforms in the negative regulation of the mouse ppTRH gene in CV-1 cells. These results were consistent with our previous findings in the negative regulation of the human ppTRH gene promoter using rat and human T₃R isoforms in a human neuroblastoma cell line (6). However, the results in our studies contradicted those obtained by others, demonstrating that TBOX preferentially exerts inhibitory regulation of human and rat ppTRH gene transcription by T₃ compared with TRα1 in CV-1 cells and in primary culture of chick hypothalamic neurons, respectively (5, 7). The discrepancy of these results may be explained by three reasons: 1) the contents of endogenous T₃R and RXR may differ among these cell lines, 2) the mechanism involved in the negative regulation by T₃ appeared to be distinct between the human and mouse ppTRH gene, and 3) T₃R isoforms in different species may have distinctive functions by their structural differences.

In summary, the mouse ppTRH gene promoter was negatively regulated by T₃, probably through collaboration of T₃R monomers with an uncharacterized factor interacting with the proximal promoter-downstream element. The characterization of such DNA binding factor(s) may provide a new concept to elucidate the molecular mechanism by which the T₃/T₃R complex negatively regulates gene transcription.

Acknowledgments—We thank Dr. Ronald Evans for providing us pEA101 and pBSRKR. We also thank Dr. William M. Wood for providing pSV5Trα1, β1, and β2 constructed by Dr. Virginia Sarapura.

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