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Negative Regulation of Expression of the Pituitary-Specific Transcription Factor GHF-1/Pit-1 by Thyroid Hormones through Interference with Promoter Enhancer Elements

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Expression of the growth hormone gene is due to the presence of the pituitary-specific transcription factor GHF-1/Pit-1. The action of the thyroid hormone T3 is mediated by nuclear receptors that regulate transcription by interaction with DNA elements located near promoters of the regulated genes. In this study, we show that T3 inhibits expression of the GHF-1/Pit-1 gene in rat pituitary GH4C1 cells by a novel mechanism that involves transcriptional interference with other regulatory elements of the promoter. Sequences between bp −90 and −200 of the rat GHF-1/Pit-1 gene which do not contain a hormone response element but contain two cyclic AMP-responsive elements mediate most of the repressive effect of T3. The hormone reduces basal levels of GHF-1/Pit-1 promoter activity and antagonizes its response to cyclic AMP and the tumor promoter TPA (12-*O*-tetradecanoylphorbol-13-acetate). A similar repression is found with a heterologous promoter that contains four copies of the cyclic AMP-responsive element motif. This regulation provides a novel example of the cross-talk between the thyroid hormone receptor and the signal transduction pathways used by different hormones and growth factors. Additionally, T3 interferes with in vitro binding of GHF-1/Pit-1 to a positive autoregulatory element located at bp −45 to −63 and has a detectable inhibitory effect on the activity of a promoter construct which extends to bp −90 of 5′-flanking DNA. The regulation of the transcription factor provides a novel example of negative transcriptional regulation by thyroid hormones.

The tissue-specific expression of the prolactin and growth hormone (GH) genes is dependent upon the presence of the pituitary-specific transcription factor GHF-1/Pit-1. The proximal promoters of both genes contain related regulatory elements to which this factor, a member of the homeobox POU family of DNA-binding proteins, binds with high affinity (8, 9, 12, 19, 26, 29). Analysis of the GHF-1/Pit-1 gene promoter indicates the presence of an element highly homologous to the GHF-1/Pit-1 binding sites in the GH and prolactin promoters which mediates positive autoregulation of its own expression (34) and a negative element that attenuates its expression (14). The GHF-1/Pit-1 gene promoter also contains two cyclic AMP (cAMP)-responsive elements (CREs) (14, 34) which mediate transcriptional actions of cAMP (34) and are binding sites for the cAMP-responsive transcription factor CREB (36, 37, 56).

Thyroid hormone is an important regulator of GH production both in vivo and in cultured pituitary somatotrophic cells (22, 33, 50, 55). Thyroid hormone actions are mediated by nuclear receptors, the product of c-*erbA* proto-oncogenes, which function as ligand-inducible transcription factors by binding to response elements which are normally located in the 5′-flanking region of regulated genes (10, 24, 58). In addition to mediating cell-specific expression, the GH gene promoter also contains the *cis* elements modulating transcription in response to the thyroid hormone triiodothyronine (T3). Sequences between bp −167 and −190 function as a thyroid hormone response element (TRE) and mediate stimulation by T3 (10).

In addition to stimulating transcription, the nuclear receptors can also selectively inhibit the expression of certain genes. Negative regulation of transcription can involve binding to negative response elements, as in the case of the regulation of thyroid-stimulating hormone gene expression by T3 (5, 13, 16, 17, 39, 54), but it can also be a consequence of interference with binding sites for other regulatory proteins in the regulated promoter (1, 18, 25, 36, 45) or even result from a direct interaction with other nuclear proteins, such as the components of the AP-1 complex (27, 31, 38, 40, 47, 57, 60), without requiring binding to DNA.

In this study we have examined the possibility that besides binding to the TRE, the T3 receptor could influence rat GH gene transcription by modulating GHF-1/Pit-1 gene expression. Our results show that, contrary to its effect on GH gene expression, T3 decreases GHF-1/Pit-1 mRNA and protein levels in pituitary GH4C1 cells. This decrease is a consequence of an inhibition of the activity of the GHF-1/Pit-1 promoter despite the lack of a TRE in this region and most likely results from transcriptional interference with the CREs as well as from interference with binding of the transcription factor to the autoregulatory element. These results exemplify a novel cross-talk between the nuclear receptor pathway and the membrane signal transduction pathway, leading to negative transcriptional regulation by thyroid hormones.

MATERIALS AND METHODS

Plasmids. GHF-1-CAT constructs containing progressive 5′ deletions (bp −400, −200, and −90) of the rat GHF-1/Pit-1 promoter have been described previously (34). In the plasmids −400rGHF1-CAT-ΔCREP, −400rGHF1-CAT-ΔCRED, and −400rGHF1-CAT-ΔCREP,D, the proximal CRE, the distal CRE, and both CREs, respectively, were deleted. The −400rGHF1-CAT-ΔCREP construct was obtained by linearization with *Bsi*WI, digestion with S1 nuclease, and religation. This treatment deletes 4 bp (GTAC) from the proximal CRE (GTACGTCA) located at bp −150 to −157. For construction of the distal and double CRE mutants, a *Pst*I-*Sal*I fragment (bp −430 to +15) from the native construct or the ΔCREP mutant was subcloned into pBluescript, digested with *Aat*II, blunt ended with T4 DNA polymerase, religated, and subcloned again in the parental construct. This process deleted another 4 bp (ACGT) from the distal CRE (TGACGTCA) located at bp −193 to −200. The deletions were

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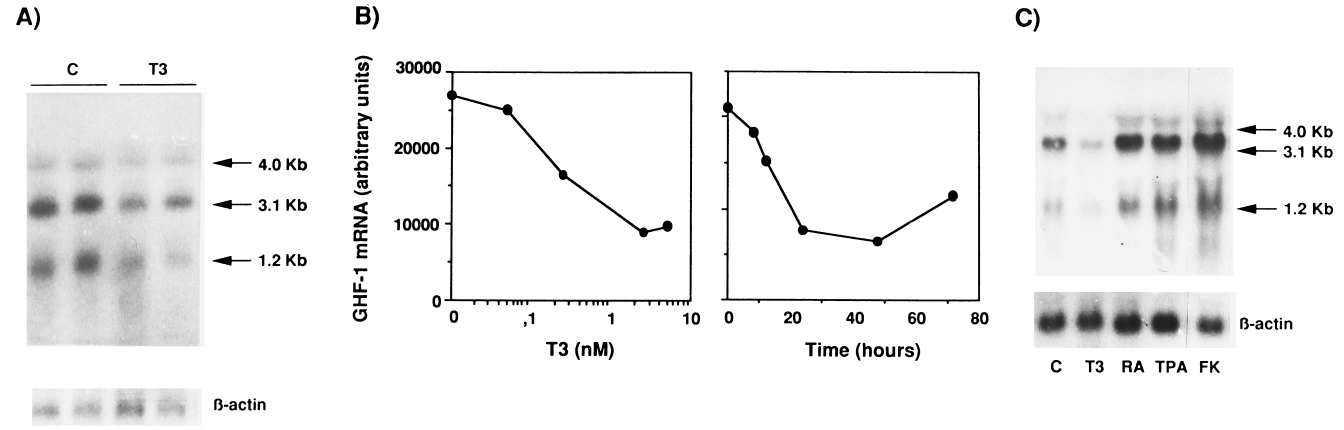

FIG. 1. Northern blot analysis of GHF-1/Pit-1 mRNA in GH4C1 cells. (A) The cells were incubated in thyroid hormone-depleted medium, and Northern blot analysis was performed with 10 μg of total RNA prepared from control cells (C) and from cells incubated with 2 nM T3 for 48 h. The blots were hybridized with a rat GHF-1 cDNA probe and with a β-actin probe as described in Materials and Methods. The sizes of the GHF-1/Pit-1 transcripts are indicated. (B) GH4C1 cells were incubated in hormone-depleted medium with increasing concentrations of T3 for 48 h (left panel) or with 2 nM T3 for the times indicated (right panel). GHF-1/Pit-1 mRNA levels were quantified by densitometric scans of Northern blots hybridized sequentially with a GHF-1/Pit-1 cDNA probe and a β-actin probe. The results were corrected by the amount of β-actin mRNA. (C) Northern blot analysis was performed with 15 μg of total RNA extracted from control cells (C), from cells incubated with 2 nM T3 or 10 μM RA for 48 h, or from cells incubated with 10 μM forskolin (FK) or 100 nM TPA for 4 h.

confirmed by DNA sequencing. The construct −90.1rGHF1-CAT, from which the positive autoregulatory element was deleted, has been previously described by McCormick et al. (35). The construct p4xCREtk-CAT is derived from a pBLCAT2 variant (30) and contains four copies of a synthetic CRE inserted into the multiple cloning site in front of the thymidine kinase (TK) promoter of pBLCAT. The CRE sequence is the one present in the c-*fos* gene at bp −60. The vectors for the retinoic acid (RA) and T3 receptors contain the cDNA sequences of the α form of the human RA receptor (RAR) (53) or the α form of chick c-*erbA*, a thyroid hormone receptor (TR) (23) under the control of the constitutively active Rous sarcoma virus (RSV) promoter. An expression vector for the CRE-binding protein CREB (36) also has the RSV promoter.

RNA extraction and hybridization. Total RNA was extracted with guanidinium isothiocyanate (15). The RNA was run in 1% formaldehyde-agarose gels, transferred to nylon membranes (Nytran), and hybridized with a 450-bp *Eco*RI fragment of a rat GHF-1 cDNA (9) labeled by random oligonucleotide priming. Hybridizations were done at 42°C with 50% formamide, and the more stringent wash was done at 55°C with 0.1× sodium dodecyl sulfate–1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). After hybridization, the ^{32}P-labeled probe was stripped off and the blot was rehybridized with a β-actin probe to correct for the amount of RNA applied. The Northern (RNA) blots were quantitated by densitometric scanning of the autoradiographs, and the GHF-1/Pit-1 mRNA data were corrected by the β-actin mRNA levels.

Cell culture and transfections. GH4C1, 235-1, Cos-7, and HeLa cells were cultured in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum and were transfected by electroporation as previously described (6, 22). Ten micrograms of the reporter plasmids was mixed with 20 million to 30 million cells and exposed to a high-voltage pulse (200 to 250 V, 960 μF) by using a Bio-Rad electroporator with a capacitor extender. The cells from each electroporation were split into different culture plates in Dulbecco modified Eagle medium containing 10% AG1x8 resin-charcoal-stripped newborn calf serum. Treatments with RA, T3, and/or TPA (12-*O*-tetradecanoylphorbol-13-acetate) and forskolin were administered at the concentrations and the times indicated in the text in this hormone-depleted medium. Each treatment was performed at least in duplicate cultures that normally showed less than 5% variation in chloramphenicol acetyltransferase (CAT) activity. CAT activity was determined as previously described (6, 22) by incubation of the cell extracts with [^{14}C]chloramphenicol. The unreacted [^{14}C]chloramphenicol and acetylated [^{14}C]chloramphenicol were separated by thin-layer chromatography, identified by autoradiography, and quantitated. Each experiment was repeated at least two to three times, with similar relative differences in regulated expression.

Western blot (immunoblot) analysis. One hundred micrograms of GH4C1 cell extracts was run in a 12% acrylamide gel and transferred to a nitrocellulose membrane. Nonspecific binding was blocked with 1% nonfat dried milk in Tris-buffered saline–0.05% Tween 20 at room temperature. The membrane was rinsed, incubated with a 1/100 dilution of GHF-1/Pit-1 antibody (10) for 3 h, washed again, and incubated with ^{125}I-labeled protein A.

Immunofluorescence. GH4C1 cells treated with T3 and RA were fixed with 4% paraformaldehyde, washed with phosphate-buffered saline–glycine, and treated with 0.5% Triton X-100 for 8 min. The cells were incubated with a nonimmune serum for 5 min to avoid nonspecific binding. The GHF-1 antibody was added for 1 h, and a second antirabbit antibody complemented with rhodamine was used as a marker.

In vitro transcription and translation. DNAs were linearized with the appropriate enzymes, and the transcription reaction was carried out by following the instructions of the mCAP RNA capping kit from Stratagene. Translation reactions were performed with a rabbit reticulocyte system lacking methionine from Promega Corporation, using 4 μCi of [^{35}S]methionine (Amersham).

Mobility shift assays. Gel retardation analyses were carried out with nuclear extracts from GH4C1, HeLa, or Cos-7 cells. The nuclear extracts were obtained by the method of Andrews and Faller (2). We used as probes oligonucleotides corresponding to the proximal GHF-1/Pit-1 binding site of the rat GH promoter (5′-CCAGCCATGAATAAATGTATAAGGG-3′), to the positive autoregulatory element in the GHF-1/Pit-1 promoter (5′-AGCTTACATGTATAAATGG ATTTCCG-3′), to a consensus CRE (5′GGATCCGAGCCCTGACGTTTACA CGAGTCAAGCTT-3′), to a consensus Sp-1 site (5′-GATGTGTGGGAGGAG CTTCT-3′), or to the TRE, TRE$_{PAL}$ (5′-AGCTCTAGGTCATGACCTGA-3′). For the binding assays, the extracts were incubated on ice for 15 min in a buffer (20 mM Tris-HCl [pH 7.5], 75 mM KCl, 1 mM dithiothreitol, 5 μg of bovine serum albumin [BSA], 13% glycerol) containing 3 μg of poly(dI-dC) and then were incubated for 15 to 20 min at room temperature with approximately 70,000 cpm of double-stranded oligonucleotide end labeled with [^{32}P]ATP, using T4 polynucleotide kinase. For competition experiments, an excess (50:1) of unlabeled double-stranded oligodeoxynucleotides was added to the binding reaction mixture. DNA-protein complexes were resolved on 5% polyacrylamide gels in 0.5% Tris-borate-EDTA buffer. The gels were then dried and autoradiographed at −70°C.

Cos-7 cells were grown in Dulbecco modified Eagle medium containing 10% fetal calf serum and were transfected by electroporation with 10 μg of expression vectors for c-*erbA* (23) or RAR (52). Each binding reaction mixture contained the same amount of Cos nuclear extract, which was obtained by adding the appropriate amount of nuclear extract from Cos cells transfected with 10 μg of an empty noncoding expression vector (RSV-0).

RESULTS

Regulation of GHF-1/Pit-1 mRNA levels in GH4C1 cells. Total RNA (10 μg) prepared from pituitary GH4C1 cells was probed with rat GHF-1/Pit-1 cDNA. Figure 1A shows results of a representative experiment using control cells and cells treated with 2 nM T3 for 48 h. Northern analysis with the GHF-1/Pit-1 probe yielded three major signals corresponding to 1.2, 3.1, and 4 kb; the most abundant form corresponded to the 3.1-kb mRNA form. Treatment with T3 did not alter β-actin mRNA. Densitometric scans from different experiments showed that T3 reduced by two- to threefold the abundance of

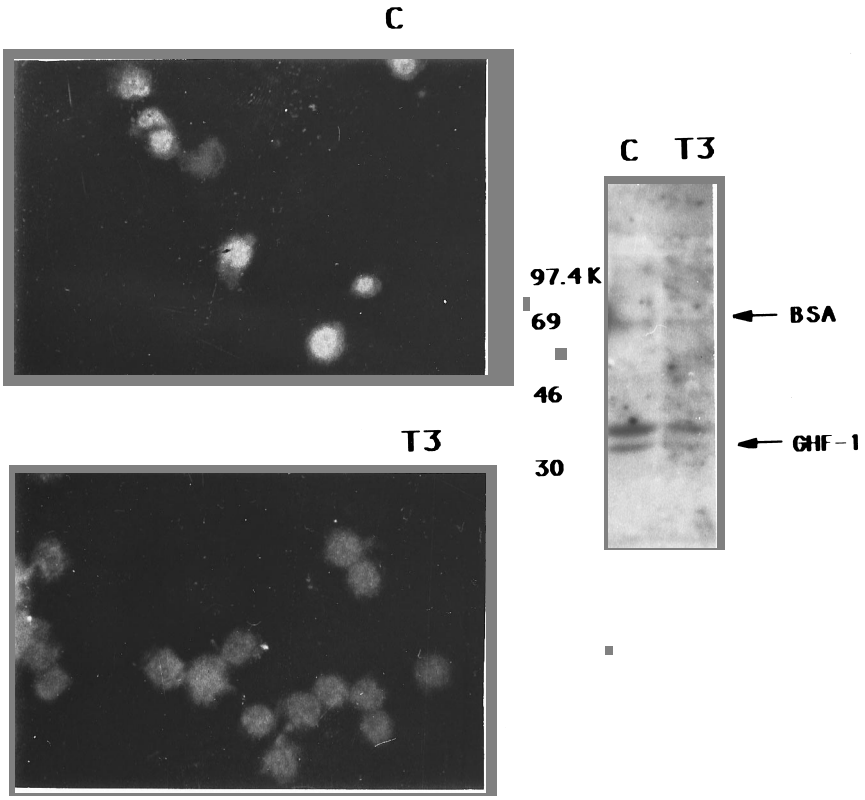

FIG. 2. GHF-1/Pit-1 protein expression in GH4C1 cells. (Left panels) Immunofluorescence assays. The cells were incubated in the presence or in the absence of 2 nM T3 for 48 h, fixed, and analyzed by indirect immunofluorescence with an anti-GHF-1 antibody as described in Materials and Methods. (Right panel) Western blot analysis of GHF-1/Pit-1. Nuclear extracts (100 μg) from control GH4C1 cells (C) and from cells treated with 2 nM T3 for 48 h were probed with the same antibody, incubated with ^{125}I-labeled protein A, and submitted to autoradiography. The antibody shows some cross-reactivity with BSA. The GHF-1/Pit-1 doublet and BSA are indicated.

the different forms which were, therefore, similarly decreased by the hormone.

Figure 1B shows the effect of a 48-h incubation with increasing concentrations of T3 on GHF-1/Pit-1 mRNA levels. The hormone produced a dose-dependent decrease of the mRNA levels. This effect was obtained at physiological hormone concentrations, since a half-maximal inhibition was found at 0.1 to 0.2 nM, a concentration identical to the estimated K_d of the T3 nuclear receptors. The maximal inhibition (two- to threefold) was obtained with 2.5 nM T3, which saturates the receptors. Figure 1B also shows the time course of the effect of 2 nM T3 on GHF-1/Pit-1 mRNA levels. The decrease was detectable after 8 to 12 h and was maximal between 24 and 48 h, at which time, in agreement with the data presented in Fig. 1A, it produced an approximately 2.5-fold reduction.

RA regulates gene expression by binding to nuclear receptors highly homologous to the T3 receptors (21). We have previously shown that RA regulates expression of the GH gene and that the TRE also mediates transcriptional regulation of this gene by RA (6). Figure 1C compares the effects of T3 and RA on GHF-1/Pit-1 mRNA levels. As can be observed, RA did not decrease but, rather, increased the levels of GHF-1/Pit-1 transcripts. On the other hand, Fig. 1C also shows the effect of a 4-h incubation with 10 μM forskolin, which increases intracellular cAMP, on GHF-1/Pit-1 transcripts. As a consequence of the existence of two binding sites for CREB in the GHF-1/Pit-1 promoter (14, 34), forskolin significantly increased GHF-1/Pit-1 mRNA levels. Since these sites may also be recognized by the AP-1 complex (44, 46), whose expression is stimulated by protein kinase C, we also examined the influence of the tumor promoter TPA on GHF-1/Pit-1 gene expression. Incubation with 100 nM TPA for 4 h increased by approximately fourfold GHF-1/Pit-1 mRNA levels, thus showing that not only the protein kinase A pathway but also the protein kinase C pathway is implicated in the regulation of this gene.

Influence of T3 on GHF-1/Pit-1 protein levels. Figure 2 shows that as a consequence of the reduced mRNA levels, the concentration of the transcription factor was also decreased in GH4C1 cells treated with T3. The levels of GHF-1/Pit-1 protein were examined by Western blot analysis and immunofluorescence assays. Protein extracts of control and T3-treated GH4C1 cells were assayed by using a specific anti-GHF-1 antibody (8). These assays (Fig. 2, right panel) revealed that the concentration of the expected 33-kDa protein doublet of immunoreactive GHF-1/Pit-1 protein was significantly reduced in the cells incubated with T3 (40 to 60% in two independent experiments). This decrease was confirmed by immunostaining (Fig. 2, left panels). In control GH4C1 cells, the nuclei appear strongly stained, whereas the cytoplasm is barely visible. However, after T3 treatment, the intensity of the nuclear staining is strongly reduced, indicating a reduction of GHF-1/Pit-1 protein.

Regulation of the activity of the GHF-1/Pit-1 promoter by T3. To examine whether the T3-receptor complexes elicit a direct control of transcription of the GHF-1/Pit-1 gene through sequences located in the 5′-flanking region of the gene, transient gene expression was performed with recombinants expressing CAT. Figure 3 shows the influence of T3 on the activity of the −400rGHF1-CAT, −200rGHF1-CAT,

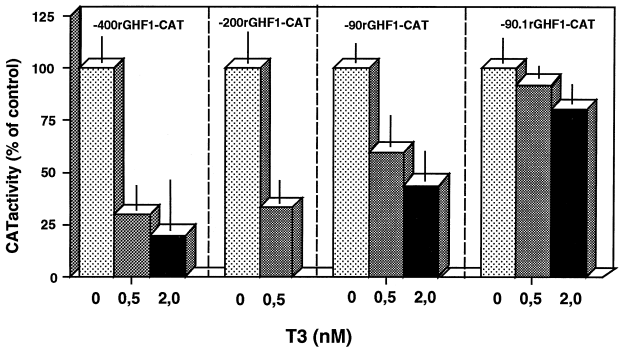

FIG. 3. Influence of T3 on GHF-1/Pit-1 promoter activity. Cells were transfected by electroporation with 10 μg of the −400rGHF1-CAT, −200rGHF1-CAT, −90rGHF1-CAT, and −90.1rGHF1-CAT constructs and incubated in medium containing 0, 0.5, or 2 nM T3. After 48 h, the cells were harvested and CAT activity was assayed in the cell lysates. CAT activity determined by the conversion of [^{14}C]chloramphenicol to its acetylated forms is illustrated. The data are expressed as the percentage of the activity of the corresponding untreated cells and are means ± standard deviations (SD) for three different transfections performed in duplicate.

−90rGHF1-CAT, and −90.1rGHF1-CAT constructs. T3 produced a marked decrease in the activity of the −400rGHF1-CAT plasmid which was dose dependent. A maximal inhibition was found with 2 nM T3 that decreased CAT activity by two- to fivefold in different experiments. Additionally, the regulation of construct −400rGHF1-CAT and that of construct −200rGHF1-CAT by T3 were identical, demonstrating that the first 200 bp of the 5′-flanking region of the gene mediate regulation by thyroid hormone. In fact, sequences between bp −200 and −90 were sufficient to mediate a significant transcriptional inhibition of GHF-1/Pit-1 gene expression by T3, since the effect of the hormone was markedly reduced on the plasmid extending to bp −90, although a significant inhibition was found at both hormone concentrations. The effect of T3 on the activity of a construct (−90.1rGHF1-CAT) which does not contain the positive autoregulatory element was negligible. This suggests that this element also plays a role in the repressive action of T3 on GHF-1/Pit-1 promoter activity.

To further document the inhibitory action of T3 on the −90rGHF1-CAT construct, we conducted additional experiments in which the GHF-1/Pit-1 promoter was cotransfected with a vector expressing the T3 receptor (TRα). In the absence of ligand, receptor overexpression did not induce important

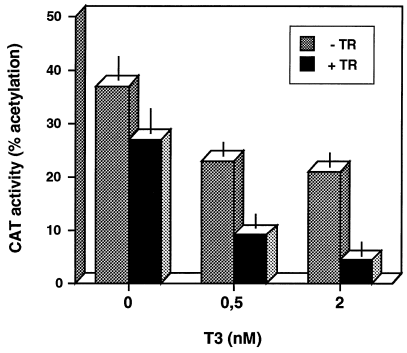

FIG. 4. Influence of overexpression of T3 receptors. GH4C1 cells were cotransfected with 10 μg of the −90rGHF1-CAT construct and 10 μg of an expression vector for the α form of the TR (+TR) or 10 μg of an empty expression vector (−TR). The data are means ± SD of CAT activity.

TABLE 1. Specificity of the negative effect of T3[a]

Construct	CAT activity (% acetylation) at indicated T3 concn		
	0 nM	0.5 nM	2.0 nM
−90rGHF1-CAT	10.5 ± 0.6	5.5 ± 0.7	4.8 ± 1.3
TATA-CAT	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
RSV-CAT	33.1 ± 2.1	30.7 ± 0.6	31.5 ± 0.9
TK-CAT	9.4 ± 0.4	15.4 ± 0.3	18.9 ± 0.8
rGH-CAT	9.0 ± 0.3	28.7 ± 2.1	33.9 ± 1.7

[a] GH4C1 cells were transfected with the −90rGHF1-CAT construct and with other recombinant plasmids expressing CAT under the control of different promoters. TATA-CAT (a minimal promoter), RSV-CAT (the Rous sarcoma virus promoter), TK-CAT (the thymidine kinase promoter), and rGH-CAT (the rat growth hormone promoter). After electroporation, the cells were grown in the absence or in the presence of T3 for 48 h and CAT activity was determined. All transfections were performed in the same experiments, and the treatments were done in duplicate. The data are means ± SD.

changes in basal CAT levels but potentiated the inhibitory effect of T3 (Fig. 4). Under these conditions, 2 nM T3 reduced promoter activity by more than fivefold.

The negative regulation of T3 is specific for this promoter, since as shown in Table 1, it does not decrease expression of CAT reporter constructs containing the RSV promoter or a minimal promoter (pTATA CAT). Additionally, in agreement with previous reports, this hormone caused a significant increase in the activity of the rat GH promoter (6, 10) and a less marked stimulation of the TK promoter (43).

Involvement of the CREs in the inhibitory effect of T3 on the GHF-1/Pit-1 promoter. Analysis of the bp −200 to −90 fragment of the GHF-1/Pit-1 promoter did not reveal the existence of a consensus TRE sequence in this region which, however, contains at bp −200 to −193 and −157 to −150 the CRE motifs. In order to examine the possible implications of these elements for the T3-mediated transcriptional inhibition, we examined the influence of T3 on the response of −400rGHF1-CAT (which contains the CREs) and constructs −90rGHF1-CAT and −90.1rGHF1-CAT (which do not contain the CREs) to forskolin and TPA. Figure 5 shows that incubation with forskolin produced a significant increase (more than fivefold) in the activity of the −400rGHF1-CAT construct and that TPA induced a stimulation at least as strong as that caused by forskolin (left panel), thus showing that the observed effect of the phorbol ester on GHF-1/Pit-1 mRNA levels is secondary to transcriptional stimulation. The effect of both compounds on promoter activity was no longer observed in the case of the −90rGHF1-CAT construct (middle panel) or the −90.1r GHF1-CAT construct (right panel), which suggests that the CREs also mediate the TPA-mediated increase.

Figure 5 also shows that treatment with T3 reduced basal promoter activity by approximately threefold. T3 strongly inhibited (from seven- to threefold) the TPA-induced activity of the CRE-containing reporter gene and, although less strongly, also reduced the response to forskolin (left panel). In agreement with the data presented in Fig. 5, the effect of the hormone on basal activity of the shorter promoter was much less marked (middle panel), and it was negligible when the autoregulatory element was deleted (right panel).

To directly assess the role of the CREs in the T3 effect, these elements were deleted both individually and together from the −400rGHF1-CAT construct. Figure 6 compares the responses of the wild-type and mutated promoters to forskolin and to TPA in the presence and in the absence of T3. The native promoter shows a marked response to TPA and forskolin (Fig.

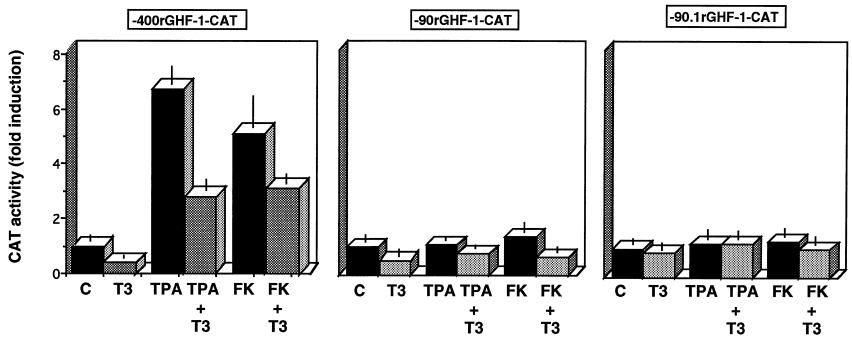

FIG. 5. T3 inhibits the response of the GHF-1/Pit-1 promoter to cAMP and phorbol esters. GH4C1 cells were used for transient transfection with the constructs −400rGHF1-CAT, −90rGHF1-CAT, and −90.1rGHF1-CAT. After electroporation, the cells were grown for 48 h in the absence or in the presence of 2 nM T3 and with 100 nM TPA or 15 μM forskolin (FK) during the last 4 h. CAT activity was quantitated and is expressed as the fold induction over the values obtained with the corresponding control (C) untreated cells. The data are means ± SD.

6A). However, as shown in Fig. 6B and C, deletion of either the proximal or the distal CRE from the −400rGHF1-CAT construct very strongly reduced TPA- and forskolin-mediated stimulation (from eight- and fivefold, respectively, to less than twofold). These results show that both elements act synergistically to increase the response to protein kinase A and protein kinase C. Activation by forskolin and TPA was totally abolished when both CREs were deleted (Fig. 6D). In agreement with the results shown in Fig. 5, T3 strongly reduced basal activity and stimulated activity of the wild-type promoter (Fig. 6A). The negative effect of T3 on basal promoter activity was markedly reduced (from more than 75 to 30% decrease) in the absence of either CRE, although the residual response to TPA and forskolin was still inhibited after incubation with the hormone (Fig. 6B and C). The negative effect of T3 on basal activity of the mutated promoter lacking both CREs (Fig. 6D) was similar to that observed in the absence of one of the individual CREs, since the hormone had a small but detectable inhibitory effect on CAT levels. This inhibition was also very similar to that observed with the −90rGHF1-CAT construct (Fig. 3 to 5).

Further proof of the involvement of the CREs in the stimulatory effect of TPA and the repression caused by T3 on GHF-1/Pit-1 promoter activity was obtained with a plasmid containing a tandem repeat of this motif (p4xCREtk-CAT) ligated to the TK promoter. Figure 7 shows that expression of this plasmid was significantly increased not only by forskolin but also by TPA, thus reinforcing the hypothesis that this response element mediates the TPA-induced increase of GHF-1/Pit-1 gene expression. In addition, the effect of T3 on the response of the construct p4xCREtk-CAT was very similar to that found with the GHF-1/Pit-1 promoter. T3 strongly reduced the response to TPA (from fourfold in the absence of hormone to almost basal levels in T3-treated cells) and to forskolin (from almost fivefold in the absence of hormone to less than threefold in the presence of T3). However, T3 repression was not observed with the CRE construct in the absence of TPA or forskolin treatment.

Influence of T3 on the interaction of nuclear proteins with CREs. In some cases, negative regulation of gene expression by nuclear receptors involves a mechanism of competitive DNA binding with other enhancer elements (1, 18, 48). However, as examined by gel retardation analysis, the T3 receptor does not bind to the CREs. Binding of in vitro translated receptor to a TRE (TREPAL) was efficiently abolished by the same sequence, whereas an excess of a CRE oligonucleotide did not decrease the intensity of the retarded bands. Similarly, binding of nuclear GH4C1 proteins to the CRE was inhibited by this element but not by TREPAL. In addition, the T3 receptor (obtained by either in vitro translation or expression in Cos cells) did not produce the appearance of retarded bands with the CRE (data not shown).

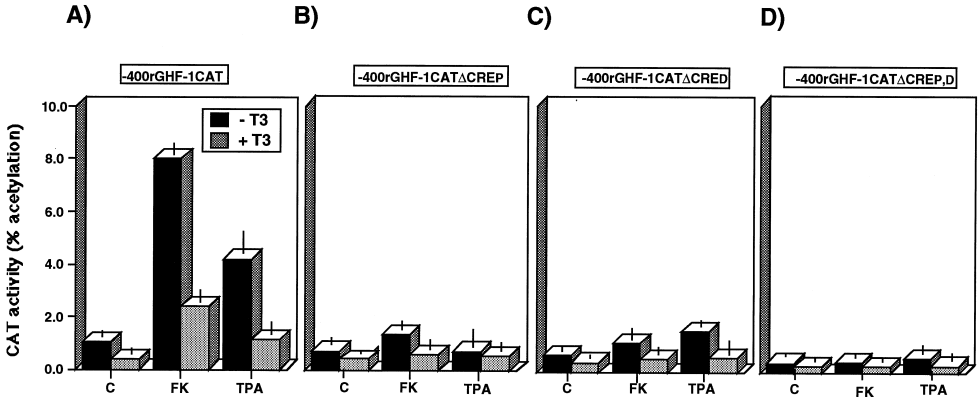

FIG. 6. Implications of the CREs for the inhibitory response to T3. The cells were transfected with 10 μg of the following constructs: −400rGHF1-CAT, which contains the native promoter (A); −400rGHF1-CAT-ΔCREP, from which the proximal CRE has been deleted (B); −400rGHF1-CAT-ΔCRED, which lacks the distal CRE (C); and −400rGHF1-CAT-ΔCREP,D, which lacks both CREs (D). The cells were incubated with TPA or forskolin in the presence or in the absence of T3 as indicated in the legend to Fig. 5, and CAT activity was determined. The data are means ± SD obtained in two independent transfections.

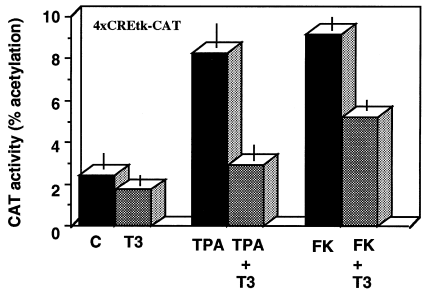

FIG. 7. Transcriptional repression of CRE sites by T3 in GH4C1 cells. CAT activity was determined in cells transfected with 10 μg of a plasmid containing a tandem repeat of CREs under the control of the TK promoter (4xCREtk-CAT). The data are means of CAT activity for duplicate transfections determined after the treatments whose results are shown in Fig. 5 and 6: cells were grown for 48 h in the presence or in the absence of 2 nM T3 and with 100 nM TPA or 15 μM forskolin (FK) during the last 4 h. C, control cells.

We next examined the possibility that the treatment with T3 could influence the abundance of nuclear proteins bound to the CRE. For this purpose, we conducted mobility shift assays with the CRE oligonucleotide and extracts from control GH4C1 cells and from cells treated with T3. As a control, we also used an Sp1 site, since the complex generated by this factor is unaffected by T3. Figure 8 illustrates that incubation of both DNA fragments with the cell extracts resulted in the formation of specific retarded bands whose intensity was not significantly reduced in the cells incubated with T3. Quantification of three independent experiments showed less than 20% reduction in CRE-binding proteins in T3-treated cells. Furthermore, the hormone did not alter the abundance of CRE-binding proteins when the cells were incubated with the combination of T3 and forskolin or TPA (data not illustrated).

Influence of overexpression of CREB on the regulation of GHF-1/Pit-1 promoter activity by T3. Since the activity of CREs is mainly dependent on the transcription factor CREB, we tested the effect of overexpression of this factor on the transcriptional interference of the hormone with the GHF-1/Pit-1 promoter. However, overexpression of CREB did not abolish the inhibitory effect of T3. Cotransfection of the −400rGHF1-CAT construct with an expression vector of this protein (RSV-CREB) further increased the effect of TPA and forskolin on GHF-1/Pit-1 promoter activity, but T3 produced a reduction of basal and stimulated expression which was quantitatively very similar to that obtained in the absence of exogenous CREB. Again, the regulation by T3 of the GHF-1/Pit-1 promoter and that of the 4xCRE-containing construct were identical, and the hormone reduced the response of the latter to forskolin and TPA also in the presence of an excess amount of CREB (data not illustrated).

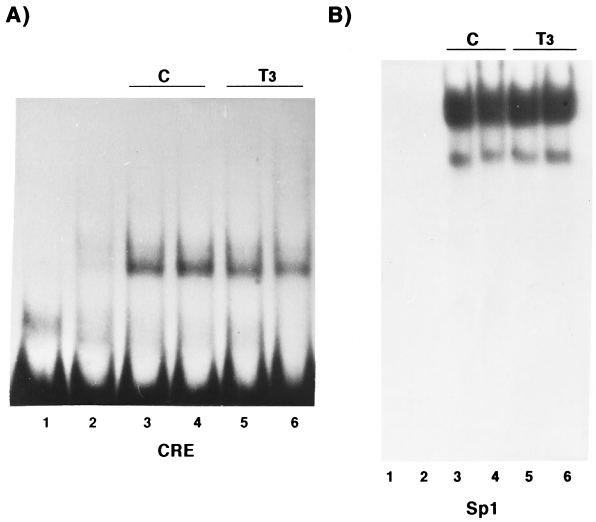

FIG. 8. Abundance of CRE-binding proteins in T3-treated cells. Nuclear extracts (6 μg) from control (C) untreated GH4C1 cells and cells treated with 2 nM T3 for 48 h were incubated with ^{32}P-labeled CRE (A) or Sp1 (B) oligonucleotides and analyzed by gel retardation assay. (A and B) Lanes 1 indicate the mobility of the free probe; lanes 2 show competition with a 50-fold excess of unlabeled oligonucleotide.

Influence of the T3 receptor on GHF-1/Pit-1 binding to DNA. Since the transactivation experiments demonstrated that T3 had a detectable effect on the activity of the −90rGHF1-CAT construct, which contains the positive autoregulatory element for the transcription factor, we next analyzed the influence of the T3 receptor on GHF-1/Pit-1 binding to DNA. As a source of GHF-1/Pit-1, we used nuclear extracts from pituitary 235-1 cells which express high levels of this factor and extremely low levels of TRs (10- to 20-fold less than GH4C1 cells) as well as proteins obtained by in vitro translation. As a control, HeLa cells that do not express the GHF-1/Pit-1 gene and have very low receptor levels were also used. Figure 9 shows the influence of the T3 receptors on GHF-1/Pit-1 binding to the autoregulatory element in the GHF-1/Pit-1 gene (Fig. 9B) and to the proximal element in the rat GH gene (Fig. 9A and C). Nuclear extracts of 235-1 cells (6 μg) produced a strong retardation with both GHF-1/Pit-1 binding sites (Fig. 9, lanes 6 and 15), whereas no protein-DNA complexes were formed with the same amount of HeLa cell extracts (Fig. 9, lanes 7 to 9), corresponding to the absence of GHF-1/Pit-1 in these cells. The addition of 5 μg of extracts from Cos cells transfected with the TR TRα produced a marked decrease in the intensity of the retarded band formed with both GHF-1/Pit-1 DNA binding elements (Fig. 9, lanes 5 and 14) in comparison with the samples that received the same amount of extracts from Cos-7 cells transfected with an empty expression vector (Fig. 9, lanes 3 and 13). The possibility that the receptor could compete with GHF-1/Pit-1 for binding to its cognate sites can be dismissed, since no retardation was observed when extracts from receptor-transfected cells were mixed with HeLa extracts (Fig. 9, lane 9). The decrease in GHF-1/Pit-1 binding is specific for the TR, since other nuclear receptors do not produce this effect. In fact, the addition of 5 μg of Cos-7 cells transfected with an expression vector for the RA receptor RARα did not decrease but rather increased the intensity of the band shift formed with the GHF-1/Pit-1 binding site of the GH promoter (Fig. 9, lane 4). These results were confirmed by using purified proteins obtained by in vitro translation. Figure 9C shows the influence of T3 receptors on binding of the transcription factor to its cognate site. The receptor preparation bound strongly to the TREPAL (not shown) and did not bind to the GHF-1/Pit-1 site (Fig. 9, lane 19). However, this preparation significantly inhibited GHF-1/Pit-1 binding (Fig. 9, lane 21). This inhibitory effect was exerted in a ligand-independent manner, since incubation with T3 did not modify this inhibition (Fig. 9, lane 22). The ligand-independent effect was also observed in an experiment similar to those described in the legend to Fig. 9A and B, in which the T3 receptor expressed in Cos-7 cells decreased endogenous binding of 235-1 cells with the same potency in the presence and in the absence of T3 (data not illustrated).

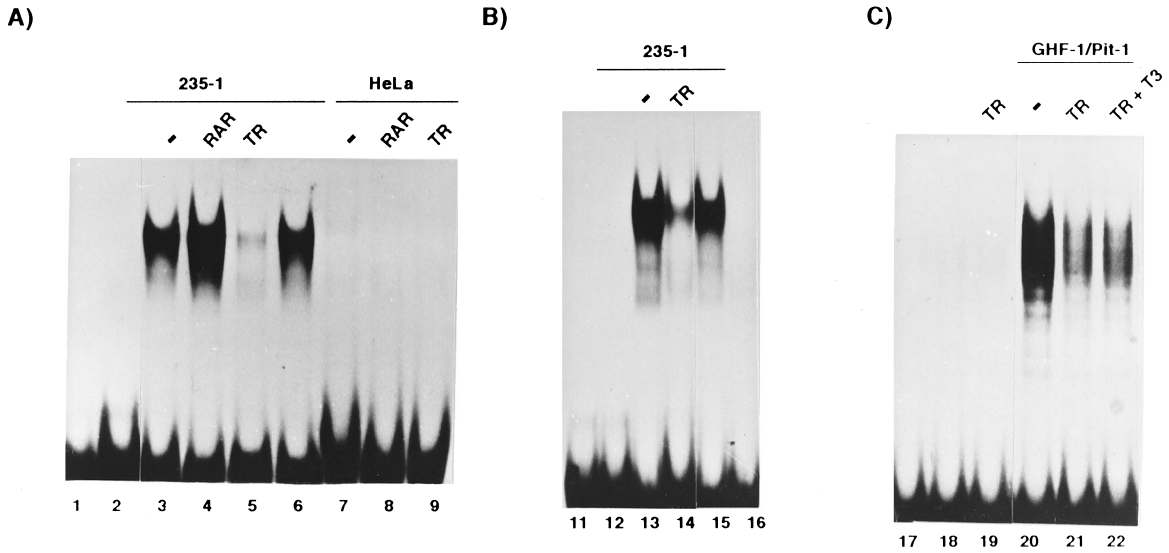

FIG. 9. The T3 receptor antagonizes binding of GHF-1/Pit-1 to DNA elements. Gel retardation analysis was performed with the positive autoregulatory element of the GHF-1/Pit-1 promoter (B) or the proximal binding element in the rat GH promoter (A and C). (A and B) Nuclear extracts (6 μg) from 235-1 cells which contain endogenous GHF-1/Pit-1 or HeLa cells which do not contain this factor were incubated with 5 μg of Cos-7 cells transfected with an expression vector for TR or RAR or with an empty expression vector (−). The reaction mixtures were incubated with the corresponding ^{32}P-labeled GHF-1/Pit-1 binding site and analyzed by the gel retardation assay. The mobility of the unretarded probes is shown in lanes 1 and 10, and lanes 2 and 12 show competition of lanes 3 and 13 with an excess of unlabeled DNA. Lanes 6 and 15 show the band shift produced by the 235-1 cell extracts in the absence of Cos-7 extracts, and lane 16 shows that Cos-7 cell extracts do not produce retardation. (C) In vitro translated GHF-1/Pit-1 (1.5 μl) was incubated with the oligonucleotide in the presence of 3.5 μl of unprogrammed reticulocyte lysate (lane 20) or the same amount of in vitro translated T3 receptor (lane 21). In lane 22, the reaction mixture of lane 20 was incubated in the presence of 100 nM T3. Neither the lysate (lane 18) nor the receptor (lane 19) retarded the probe whose mobility is shown in lane 17.

DISCUSSION

We present evidence that the thyroid hormone T3 negatively regulates the expression of the gene encoding the pituitary-specific transcription factor GHF-1/Pit-1. The dose dependence of the T3-caused decrease of GHF-1/Pit-1 mRNA levels is compatible with a TR-mediated effect, since the half-maximal inhibition was found at T3 concentrations coincident with the K_d of T3-receptor binding, and a maximal effect was obtained at the concentrations which fully occupy the receptors (45, 55). That repression of GHF-1/Pit-1 gene expression requires the presence of functional amounts of TR is confirmed by the finding that we have not observed regulation of the GHF-1/Pit-1 gene by T3 in lactotrophic 235-1 cells that have very low T3 receptor levels (unpublished observations).

The inhibition of GHF-1/Pit-1 expression by T3 was rather unexpected, since thyroid hormone has been shown to transcriptionally stimulate expression of the GH gene (54), which, on the other hand, absolutely requires the presence of this factor to be transcribed in pituitary cells (19, 26, 35, 49, 53). However, even though a major function of T3 in somatotrophic cell function is the stimulation of GH gene expression, in vivo observations with rats show that in the hyperthyroid state there is not an increase in pituitary GH content above control euthyroid levels. In fact, we have observed that chronic hyperthyroidism leads to a decrease in GH content (41). Data obtained by using cultured pituitary cells also show a rapid increase in GH transcription rate which is maximal after 4 h of incubation with T3 and then decreases (55). Although this reduction has been attributed to a partial depletion of TR levels by its own ligand, our data suggest that at longer incubation times a decrease in GHF-1/Pit-1 gene expression could also contribute to the lower velocity of GH gene transcription. The evidence that thyroid hormone has a dual effect on GH gene expression—inducing transcription by binding to the TRE present in the promoter region and interfering with the synthesis of the transcription factor binding to this region—suggests the existence of a negative regulatory mechanism which could attenuate the stimulation of the GH gene in the presence of excess amounts of thyroid hormones.

Negative regulation of the GHF-1/Pit-1 gene by T3 occurs at a transcriptional level and is mediated by sequences located in the 5′-flanking region of the gene. We have investigated the mechanism by which T3 negatively regulates expression of the transcription factor. The data presented here suggest a mechanism that does not involve interaction between the receptor and a DNA-responsive element, since the fragment of the promoter that mediates the T3 effect does not contain any of the previously identified negative TRE sequences that mediate transcriptional repression by thyroid hormone (39). Thus, T3 could repress GHF-1/Pit-1 gene expression by interfering with transcriptional activation induced by other factors that bind to the promoter. This promoter contains a GHF-1/Pit-1 binding site which mediates the positive autoregulation of its own expression and two CREs (34). A fragment of the promoter which contains only the GHF-1/Pit-1 binding site shows some negative regulation by T3, but the fragment containing the CRE sequences is much more strongly repressed by the hormone. In consequence, the CREs were good candidates to be involved in the negative effect of thyroid hormone. That this is indeed the case is suggested by the finding that T3 significantly antagonizes the induction of GHF-1/Pit-1 promoter activity by cAMP and phorbol esters. The existence of extensive cross-talk between the protein kinase A and C pathways is well documented (7, 11, 32, 46, 59), and our data show that stimulation of GHF-1/Pit-1 gene expression by TPA is not mediated by a tumor promoter response element or AP-1 binding site (3) but rather by the CRE sequences that mediate transcriptional regulation by cAMP. Direct proof of the involvement of these motifs in the regulation by T3 and TPA was obtained with mutant promoters from which the CREs have been deleted.

Deletion of either CRE greatly decreases the inhibitory effect of T3 and profoundly inhibits stimulation by TPA or forskolin. These findings have also been confirmed with a heterologous promoter containing a tandem of CRE sequences. The tumor promoter stimulated at least as strongly as cAMP its activity in GH4C1 cells, and T3 had a repressive effect which was identical to that caused in the GHF-1/Pit-1 promoter. Taken together, our results stress the importance of the CREs in GHF-1/Pit-1 gene expression, since in these elements the positive regulation by the protein kinase A and C pathways and the negative regulation by the TRs converge. The CREs have also been recently shown to mediate the effects of the glucocorticoid receptor in the regulation of GHF-1/Pit-1 promoter activity (28). Phorbol esters act in combination with glucocorticoids to enhance expression from this promoter, and sequences containing both CREs are required for this cooperation.

The T3 receptor could cause transcriptional interference with the activity of the CREs through direct DNA-protein or protein-protein interactions. Our DNA binding experiments indicate that the T3 receptor does not bind directly to the CRE sequences. These results, in which the T3 receptors antagonize transcriptional stimulation mediated by CRE sequences without binding to these elements, are reminiscent of previous observations obtained with AP-1 binding sites. The T3 receptor and the AP-1 complex antagonize each other's activity by a mechanism that does not require protein-DNA interaction but rather appears to involve a direct protein-protein interaction (60). A possible interpretation of our results would be the existence of interaction between the receptors and the transcription factor CREB, although direct proof of this mechanism is still lacking. However, Jun (heterodimerizing with CREB) and the CCAAT/enhancer-binding protein (C/EBP) also recognize CRE sequences (4, 42), and these proteins could, therefore, participate in this interaction. Mutual antagonistic effects of CREB and the glucocorticoid receptor have been previously described (1, 13, 20, 51). This repression had been attributed to the presence of overlapping binding sites for CREB and the receptors in the regulated promoter (1). However, more recent reports suggest that the repression is caused by receptor interactions with CREB or associated transcription factors (13) or even by competition for a mutually required target protein (51).

T3 could act more indirectly by influencing the expression of the CRE-binding proteins. However, our experiments using gel retardation analysis also show that there is not a decrease in the total concentration of CRE-binding proteins in the cells incubated with the hormone and that, additionally, an excess of CREB does not reverse this inhibition. It remains possible that the thyroid hormone could antagonize the effect of cAMP or phorbol esters by modulating the activity (i.e., by changes in the phosphorylation) of the transcription factors bound to the CREs.

Our results also demonstrate that the autoregulatory element plays a role in the negative regulation of GHF-1/Pit-1 promoter activity by T3. Deletion of this element from a construct which also lacks the CREs abolishes T3-mediated repression. The finding that the hormone still has an inhibitory effect on the activity of the −90rGHF1-CAT construct is consistent with a reduced stimulation of this autoregulatory element by the decreased endogenous levels of the transcription factor in hormone-treated cells. Additionally, in vitro incubation of nuclear cell extracts with T3 receptors results in a significant decrease in GHF-1/Pit-1 binding to its cognate DNA element, which can contribute to the observed regulation. The presence of the ligand is not required for this in vitro effect of the receptor, suggesting the existence of hormone-independent mechanisms in this regulation. Since the receptor does not bind to this sequence, a protein-protein interaction between the T3 receptor and the transcription factor, leading to a loss of binding of GHF-1/Pit-1, as occurs with the receptor and the AP-1 complex (60), is the most likely explanation for these observations.

In conclusion, by our investigation of the regulation of the transcription factor GHF-1/Pit-1 we have provided evidence for a novel negative regulatory function of the TR that is not mediated by a classical mechanism of binding to a DNA-responsive element but rather involves transcriptional interference with other regulatory elements. The transcriptional repression of this gene provides a new example of the cross-talk between the nuclear receptors and the membrane signal transduction pathway and indicates the complexity of this interaction that includes both protein kinase A- and protein kinase C-dependent mechanisms.

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