Thyroid Hormone Inhibits the Human Prolactin Gene Promoter by Interfering with Activating Protein-1 and Estrogen Stimulations

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Transcription of the human PRL (hPRL) gene in the pituitary is subject to tissue-specific and multihormonal regulation involving two main regulatory regions, a proximal promoter and a distal enhancer.

In this report we show that thyroid hormone inhibits the expression of the hPRL gene in rat pituitary cells. Transient expression experiments show that thyroid hormone regulation involves a strong inhibitory element, located in the proximal (−164/−35) promoter, which is modulated by a more distal stimulatory response control region. Gel retardation experiments reveal that the thyroid hormone receptor does not bind to the proximal negative element.

We show the existence of an activating protein-1 (AP-1) response element located at positions −61 to −54 of the proximal promoter, conferring AP-1 stimulation to the hPRL promoter. This AP-1 induction is abolished when hormone-bound thyroid hormone receptor is present, indicating that there is an interference between the thyroid hormone receptor and AP-1 regulatory pathways.

Furthermore, using the complete hPRL upstream region, we show that estrogen induction is abolished by simultaneous thyroid hormone treatment. (Molecular Endocrinology 11: 986–996, 1997)

INTRODUCTION

In vertebrates, thyroid hormone (T_3) regulates a vast array of biological processes, including metamorphosis, development, growth, metabolism, and homeostasis, mainly through specific regulation of gene transcription (1). The effects of T_3 are mediated by thyroid hormone receptors (TRs) belonging to the large nuclear hormone receptor superfamily (2, 3). Some members of this superfamily, such as the estrogen and glucocorticoid receptors, bind to conserved hormone response elements (AGGTCA and AGAACA, respectively) in the promoter regions of target genes. The binding of TR to thyroid hormone response elements (TREs) is apparently more complex. TREs vary considerably in nucleotide sequence and with regard to the number, spacing, and arrangement of binding sites (4–7). Most TREs described to date are composed of two or more copies of the motif (A/G)GG(A/T)CA (or a related sequence) arranged as palindromes or direct repeats. The spacing of these half-sites is critical for TR homodimer formation or heterodimerization with other members of the nuclear receptor superfamily, such as the retinoic acid (RAR and RXR) and vitamin D receptors (8). Most well known TREs mediate positive regulation by thyroid hormone. Negative T_3 response elements (nTREs) have been less well characterized, being sometimes described as half-site sequences to which TR would bind as a monomer (9). Functional interactions between various nuclear receptors and between such receptors and other transcription factors are well documented (10–14).

Recently, thyroid hormone receptors have been shown to inhibit gene expression by interfering with the activity of the activating protein-1 (AP-1) transcription factor (15–22). This factor is a dimer whose components are respectively encoded by the *jun/fos* protooncogene family (23). It binds to a specific DNA sequence (ATGAGTCA) and stimulates gene transcription in response to activation of the protein kinase C pathway (24). TRs exert their inhibitory effect through direct interaction with the AP-1 complex in solution, leading to the formation of a transcriptionally inactive TR/AP-1 complex. TRs do not compete with AP-1 for DNA binding sites (15).

PRL is a hormone essentially secreted by the anterior pituitary. Transcription of the human PRL (hPRL) gene in the pituitary is subject to tissue-specific and multihormonal regulation involving the POU domain transcription factor Pit-1 (25–28). In the 5′-flanking sequence of the hPRL gene, two regulatory regions

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have been identified, a proximal promoter (−250/−35) containing three Pit-1-binding sites and a distal enhancer (−2000/−1200) with eight additional Pit-1-binding sites (29, 30). Regulation of hPRL gene transcription by phorbol esters, epidermal growth factor, TRH, Ca^{2+}, and cAMP depends on elements located within the first 250 bp of the promoter (31–34). Estrogens are reported to stimulate hPRL gene expression through a response element next to the distal enhancer (34). Nothing is known about T_3 regulation of hPRL, whereas contradictory results have been obtained in GH pituitary tumor cell lines for rat PRL (rPRL) T_3 regulation. T_3 was found to inhibit rPRL promoter-driven transcription (35–37) in GH_1 cells but to stimulate it in GH_4C_1 cells (38) through the same response element, located in the proximal promoter (coordinates −176/−11). From studies in GH_3 cells, two TREs were identified in the rPRL gene: a positive response element in the distal enhancer next to the estrogen receptor response element and a negative response element in the 292-bp span of the proximal promoter (39).

This report focuses for the first time on the regulation of the hPRL gene by thyroid hormone. We show that the hPRL gene promoter contains both a positive and a negative TRE. The negative effect is stronger than the positive one and involves cross-talk between TR and AP-1.

RESULTS

The hPRL Promoter Is Inhibited by Thyroid Hormone

To identify possible T_3 response elements in the 5′-flanking region of the hPRL gene, we used various constructs containing either the complete 5000-bp pituitary hPRL promoter or a 5′-deleted portion of it fused to the chloramphenicol acetyltransferase (CAT) reporter gene (29). The constructs were introduced by electroporation into GH3B6 cells (a PRL-producing pituitary cell line). Figure 1 shows the CAT activity produced by each construct in the absence and presence of T_3 as well as the inhibition factor in the presence of T_3.

A construct containing the 500-bp promoter region of the human placental lactogen B gene (hCS-B, construct p500CSBCAT) (14) was used as a positive control. In this construct, T_3 causes 4-fold stimulation of reporter gene expression (0.25-fold repression). As expected, constitutive thymidine kinase (TK)-driven CAT gene expression is unaffected by T_3 treatment. In contrast, expression from all hPRL constructs is inhibited in the presence of T_3. The shorter constructs (p250PRLCAT and p740PRLCAT) are inhibited approximately 4-fold, whereas the longer ones (p1330-PRLCAT, p1750PRLCAT, p2627PRLCAT, p3500-PRLCAT, and p5000PRLCAT) are inhibited only 2-fold. Similar results were obtained in GC cells (data not shown). These results suggest the presence of two distinct T_3 response elements in the hPRL promoter whatever pituitary cell line used. One response element, located in the 250-bp proximal promoter, would mediate a strong inhibitory effect; the second, located between coordinates −1330 and −740, would have a weaker positive effect, attenuating the inhibitory effect of the proximal region.

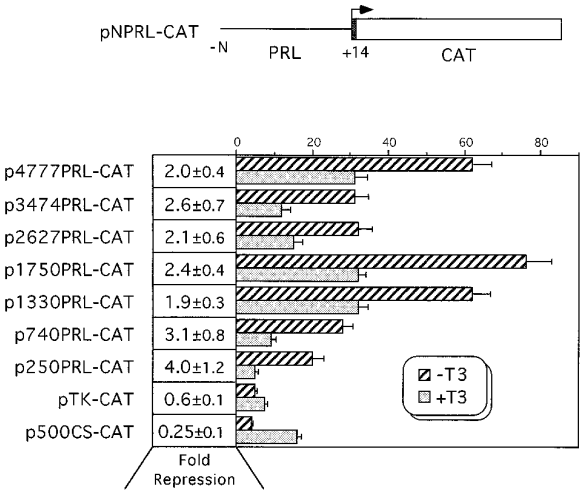

Fig. 1. Influence of T_3 on hPRL Promoter Activity

GH_3B_6 cells were transfected by electroporation with 30 μg of the indicated fusion construct. The various PRLCAT constructs carry 5′-deletions of the hPRL promoter (decreasing in length from p5000PRLCAT to p250PRLCAT). Construct p500CSBCAT carries the human placental lactogen promoter fused to the CAT reporter gene. Plasmid pTKCAT (or pBLCAT2) carries the CAT reporter gene under the control of the TK promoter. Electroporated cells were incubated for 48 h in medium with or without 10 nM T_3. Cells were harvested, and CAT activity was assayed in cell lysates. The percentage of CAT conversion in the absence (*hatched boxes*) or presence (*gray boxes*) of T_3 is shown, and the ratio between these values is indicated *to the left* as fold repression for each construct. Values are the mean ± SEM of five independent transfections, each performed in duplicate.

The Inhibitory Effect Is not Pit-1 Dependent

The first 250 bp of the hPRL promoter (proximal promoter) include three well known Pit-1-binding sites (30). They also include the A site involved in the responses to cAMP, TRH, and Ca^{2+} (32, 33) (Fig. 2a). To study T_3 regulation in this proximal region, we tested two constructs: one bearing the entire proximal region without the TATA box (bp −250/−35) and one bearing the same fragment lacking the P3 Pit-1-binding site (bp −164/−35). Both proximal promoter segments were cloned in front of the TK promoter fused with the CAT reporter gene. The constructs were introduced by electroporation into GH_3B_6 cells (Fig. 2b). As a positive control we used a construct containing three palindromic TRE sequences cloned in front of the TK-CAT fusion. As expected, T_3 stimulates CAT expression

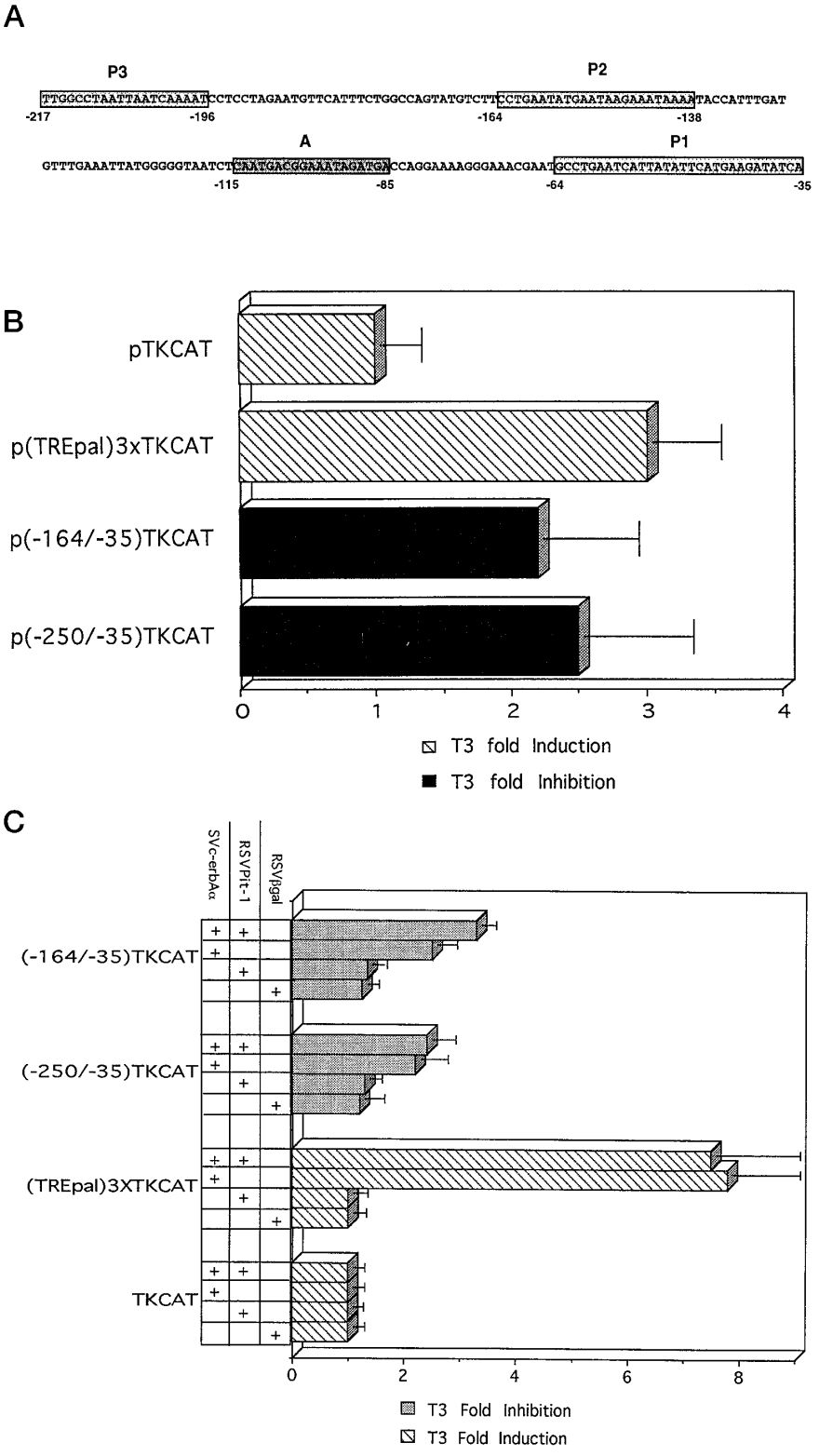

Fig. 2. T_3 Regulation of a Heterologous Promoter in Pituitary and Nonpituitary Cells

A, Sequence of the sense strand of the 217-bp hPRL promoter (57). The positions of the three proximal, high affinity Pit-1-binding sites and of the A sequence are indicated, respectively, by boxes P1, P2, P3, and A. B, GH_3B_6 cells were transfected with 15 μg of the indicated plasmid. Plasmid pTKCAT carries the TK promoter fused to the CAT reporter gene. Plasmids p(−164/−35)TKCAT and p(−250/−35)TKCAT additionally carry the indicated base pairs of the hPRL proximal promoter in front of the TK promoter; p(TREpal)3XTKCAT carries instead three copies of the TREpal sequence in front of the TK promoter. Cells were grown for 48 h in the absence or presence of 10 nM T_3. CAT activity was measured, and the data are presented as described

from this control plasmid approximately 3-fold while having no effect on the negative control (pTKCAT), indicating that T_3 effects are specific under our experimental conditions. CAT expression from both p(−250/−35)TKCAT and p(−164/−35)TKCAT is inhibited about 3-fold by T_3. This shows that the T_3 response element can mediate inhibition of the heterologous TK promoter. Our experiments further show that the nTRE is located between bp −164 and −35 of the hPRL proximal promoter.

To detect a possible involvement of Pit-1 in T_3 inhibition of the hPRL proximal promoter, we performed cotransfection experiments in which nonpituitary HeLa cells received one of the above-mentioned constructs in combination with a vector expressing either hTRα1 complementary DNA (cDNA; pSV2c-*erb*Aα), hPit-1 cDNA (pRSVPit-1), or both vectors (Fig. 2c). Expression from pTKCAT is unaffected by the presence of one or both of these expression vectors. As expected, T_3 stimulates expression from p(TREpal)3XTCAT in the presence of pSV2c-*erb*Aα; this effect is unaltered by the additional presence of pRSVPit-1. In cells receiving either p(−250/−35)TKCAT or p(−164/−35)TKCAT constructs, the presence of pSV2c-*erb*Aα is sufficient to promote T_3 inhibition of reporter gene expression, so clearly Pit-1 synthesis is not required for this effect. Furthermore, the inhibition factor is the same regardless of whether the Pit-1 expression vector is present. That Pit-1 is indeed synthesized in HeLa cells transfected with pRSVPit-1 is shown by the increased basal level of CAT expression recorded with p(−250/−35)TKCAT (4-fold stimulation) and p(−164/−35)TKCAT (3-fold stimulation). These results suggest that Pit-1 is not involved in the inhibitory effect exerted by T_3 through the proximal promoter region. We obtained similar results in other Pit-1-deficient cell lines, such as CV1 and JEG-3 (data not shown), reinforcing the conclusion that the effect of T_3 on the hPRL proximal promoter is not pituitary specific.

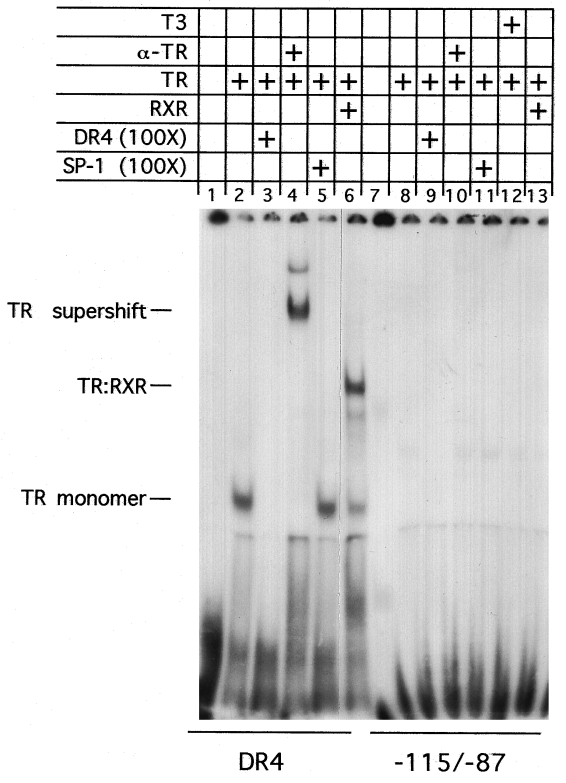

Fig. 3. TR Does not Bind to the Proximal TRE of the hPRL Promoter

Extracts of TR-expressing bacteria (4.5 μg protein) were incubated with the indicated ^{32}P-labeled DNA fragment, *i.e.* either the DR4 oligonucleotide (positive control) or the (−115/−87) sequence of the proximal hPRL promoter. The presence of competitor oligonucleotides (in 100-fold excess), anti-TR antibody (α-TR), recombinant RXR (4.5 μg), and 10 nM T_3 is indicated by a +.

Involvement of Receptor-DNA Binding in the T_3 Effects

The −164/−35 region contains two Pit-1-binding sites (P1 and P2) and the cAMP response element A (Fig. 2a). A search for potential TR-binding sites in this region revealed only one imperfect half-site, GGGTaA (−113/−108). To test the affinity of TR for this putative binding site, we performed gel retardation assays with bacterially expressed TR (Fig. 3). The probes used were an oligonucleotide containing the −115/−87 PRL proximal promoter sequence and, as a positive control, an oligonucleotide containing the DR4 consensus TR-binding sequence (two TR consensus half-sites directly repeated, spaced by 4 bp). As expected, we observed a retarded band with the DR4 oligonucleotide, corresponding to the TR monomer (Fig. 3, lane 2); this band disappeared in the presence of a 100-fold excess of cold DR4 oligonucleotide (lane 3), but not in the presence of a 100-fold excess of Sp1 oligonucleotide (lane 5). Moreover, the retarded band was supershifted in the presence of anti-TR antibodies (lane 4). All of these results prove the specificity of the

in Fig. 1B. *Black bars*, Inhibition factors; *hatched bars*, induction factors. Values are the mean ± SEM of four independent transfections, each performed in duplicate. C, HeLa cells were cotransfected by calcium phosphate precipitation with 5 μg of the indicated reporter construct and with 10 μg of each expression vector indicated. The pSV2c-*erb*Aα expresses human TRα1; pRSVPit-1 expresses human Pit-1; pRSVβgal, expressing β-galactosidase, was used as a control and to maintain a constant amount of expression vector (20 μg). Cells were incubated for 48 h in the absence or presence of 10 nM T_3, and CAT activity was measured. Inhibition factors (*gray bars*) and induction factors (*hatched bars*) were calculated as described in Fig. 1B. The data are the mean ± SEM of five independent experiments, each performed in duplicate.

retarded band. Addition of bacterially expressed RXR led to the formation of TR/RXR heterodimers (lane 6), as expected.

In contrast, TR does not specifically bind to the proximal PRL promoter sequence (−115/−87), even in the presence of RXR or T_3 (lanes 7–13). The results were the same when the probe used was the entire −164/−35 PRL proximal sequence (data not shown). This strongly suggests that the T_3 effect in this region occurs via an indirect mechanism that does not require TR binding to the DNA.

T_3 Inhibition of the hPRL Proximal Promoter Is Independent of the Forskolin Pathway, but Involves an AP-1-Binding Site

To identify the indirect T_3 repression mechanism, two alternative pathways were investigated. TR has been shown to interfere with stimulation of the PIT1 gene promoter by forskolin (40). As the proximal hPRL promoter contains cAMP response elements (−164/−35) (32, 33), we examined whether T_3 treatment might affect the response to forskolin mediated by this region. GH_3B_6 cells were transfected with a construct containing the first 164 bp of the proximal promoter cloned in front of the luciferase (LUC) reporter gene (p164PRL-LUC). The cells were treated with forskolin and/or T_3. Gene expression increased significantly (15-fold) in response to forskolin. T_3 treatment reduced the basal promoter activity approximately 2-fold. When both treatments were applied, forskolin stimulation was reduced by a factor of 2, clearly indicating that forskolin and thyroid hormone act through distinct pathways (data not shown).

In search of an alternative pathway that might be modulated by T_3, sequence analysis of the hPRL proximal promoter was performed. This analysis revealed the presence of a highly conserved AP-1 consensus motif (−61 TGAaTCAT −54) containing only one mismatch (see also Caccavelli, L., I. Manfroid, J. A. Martial, and M. Muller, in preparation). This led us to investigate whether this putative AP-1 site is functionally active and how destruction of this site affects the response of the hPRL proximal promoter to T_3. To this end, we produced a mutation in the AP-1 consensus motif of p164PRL-LUC, yielding plasmid p164(AP-1)mPRL-LUC. We then transfected GH_3B_6 cells with either p164(AP-1)mPRL-LUC, its wild-type parent, or the control plasmid pTKLUC bearing the LUC gene under the control of the TK promoter. The cells were treated or not with T_3. The results are shown in Fig. 4a. T_3 treatment did not affect expression from pTK-LUC, but it inhibited expression from p164PRL-LUC (3-fold inhibition). The mutant construct p164(AP-1)mPRL-LUC displayed 2-fold lower basal expression and did not respond to T_3.

These results show for the first time that an AP-1 consensus sequence located close to the proximal Pit-1-binding site P1 contributes to the basal activity of the hPRL promoter in pituitary cells. In addition, this

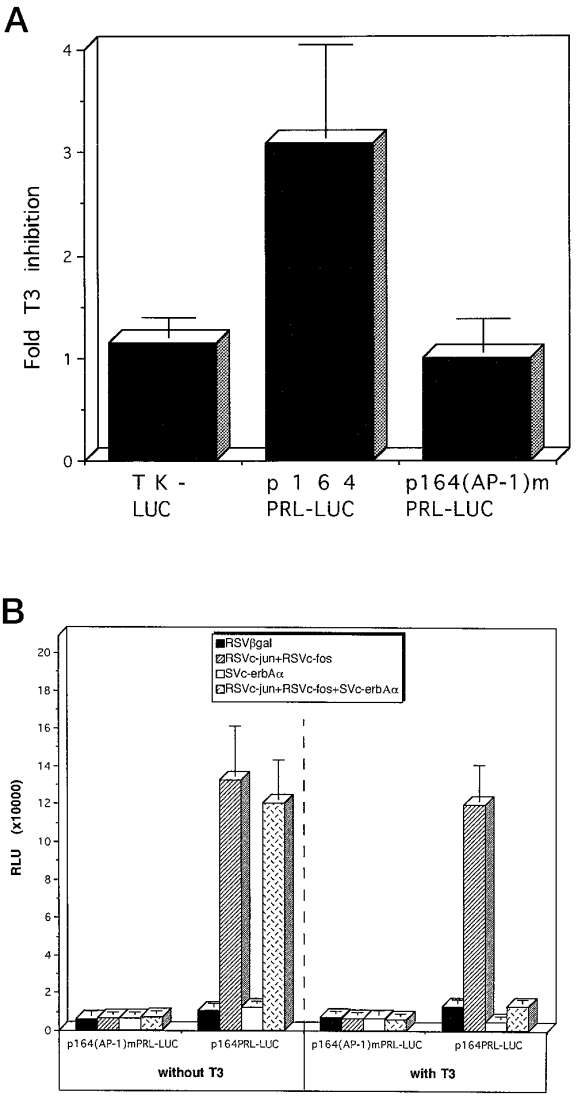

Fig. 4. T_3 Inhibits the Response of the hPRL Proximal Promoter to the AP-1 Transcription Factor

a, GH_3B_6 cells were electroporated with 10 μg p164PRL-LUC or p164(AP-1)mPRL-LUC (containing a mutated AP-1 response element). As a control we used pTK-LUC, a plasmid carrying the luciferase gene under the control of the TK promoter. The cells were incubated for 48 h with or without 10 nM T_3, and luciferase activity was measured. Inhibition factors were calculated by dividing the activity recorded for treated cells by that obtained with the corresponding untreated cells. The basal LUC activity obtained with p164(AP-1)mPRL-LUC plasmid is 2 times less than that obtained with p164PRL-LUC. The data are the mean ± SEM of three independent transfections, each performed in duplicate. b, HeLa cells were cotransfected by calcium phosphate precipitation with 10 μg p164PRL-LUC or p164(AP-1)mPRL-LUC and either 20 μg of the control expression vector pRSVβgal or 5 μg of each other expression vector indicated plus enough pRSVβgal to reach a total of 20 μg. The cells were incubated for 48 h with or without 10 nM T_3, and luciferase activity was measured. The data are the mean ± SEM of three independent experiments, each performed in triplicate.

sequence is directly involved in T_3 repression of the hPRL promoter.

T_3 Abolishes AP-1 Activation of the hPRL Promoter in Heterologous Cells

Since T_3 inhibition appears to involve a putative AP-1-binding site, we next analyzed the action of AP-1 on the hPRL proximal promoter in nonpituitary cells. We cotransfected HeLa cells with p164PRL-LUC in combination with vectors expressing c-*jun* and c-*fos* (pRSVc-*jun* and pRSVc-*fos*) and/or with pSV2c-*erb*Aα. It should be mentioned that none of these vectors has any effect on reporter gene expression from control plasmid pTK-LUC (not shown). The cells were incubated with or without T_3. Cells harboring a vector expressing β-galactosidase cDNA (pRSVβgal) were included as a control. The results are presented in Fig. 4b. In the presence of T_3, as expected, a 3-fold inhibitory effect is observed using p164PRL-LUC only when the thyroid hormone receptor is coexpressed. In the absence of TR, transcription was stimulated approximately 20-fold in the presence of c-*jun* and c-*fos* regardless of whether T_3 was present. In contrast, stimulation by c-*jun* and c-*fos* was nearly abolished when both T_3 and its receptor were present. Furthermore, mutation of the consensus AP-1 site in p164(AP-1)mPRL-LUC completely abolished both c-*jun*/c-*fos* activation and T_3 inhibition of the hPRL promoter in HeLa cells.

These results strongly suggest that AP-1 is able to activate the hPRL promoter and that T_3 exerts its inhibitory effect via an interaction between the hormone-bound receptor and the AP-1 complex.

AP-1 Binds to the hPRL Proximal Promoter

To confirm the involvement of AP-1 in hPRL expression, we performed gel retardation assays. The $-64/-35$ fragment was used as a probe in the presence of AP-1-enriched HeLa cell extracts. The results are presented in Fig. 5. One retarded complex was obtained (lane 2), which disappeared in the presence of a 100-fold excess of an oligonucleotide containing the AP-1 consensus sequence (lane 3), but not in the presence of the nonspecific DR4 oligonucleotide in a 100-fold excess (lane 4). In addition, the complex disappeared in the presence of antibodies recognizing the DNA-binding region of c-*jun* (lane 5). All of these data show that the observed band is a specific AP-1 DNA complex.

Taken together, our results confirm the following model for T_3 inhibition of hPRL gene transcription: the hormone-bound thyroid hormone receptor interacts with the AP-1 complex, thereby preventing the complex from activating hPRL gene transcription via the proximal promoter.

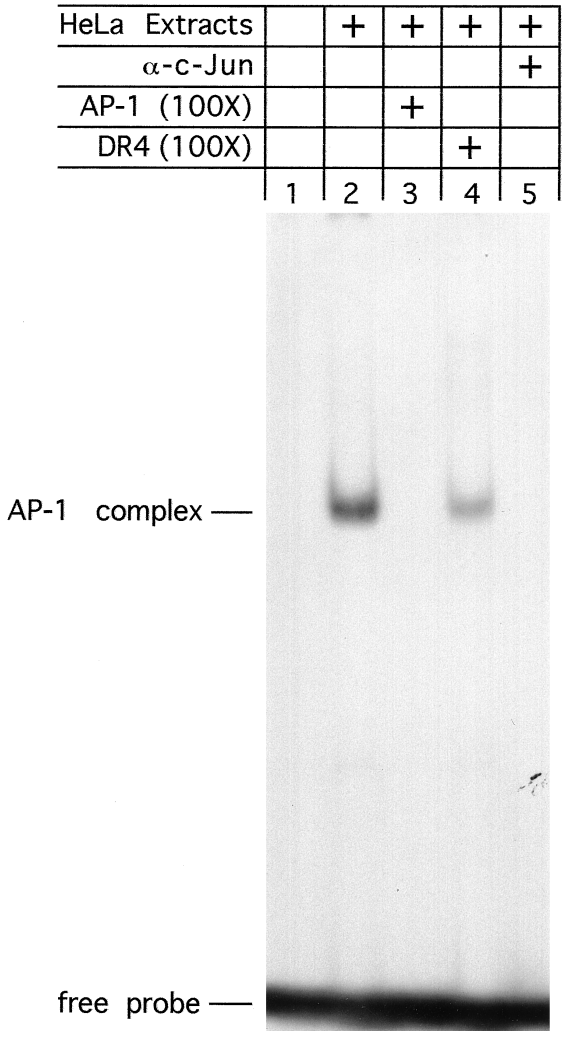

Fig. 5. AP-1 Specifically Binds to the Proximal hPRL Promoter

Total extracts from HeLa cells enriched in AP-1 factor (described in *Materials and Methods*; 5 μg) were incubated with the ^{32}P-labeled ($-64/-35$) hPRL promoter sequence and used in a gel retardation assay. Competition with a 100-fold excess of cold oligonucleotide and addition of anti-Jun antibody (α-jun) are indicated by $+$.

T_3 Abolishes Estradiol (E_2) Stimulation of the hPRL Promoter

The transfection experiments using 5′-deletion mutants of the hPRL promoter suggested the presence of a modulatory element in the distal region ($-1330/-740$) that leads to a decrease in the overall T_3 inhibition. Preliminary studies identified a complex element centered at -1200 that is able to bind TR and, in addition, contains an estrogen response element (34). To further analyze the role of this distal regulatory region, we examined the effects of thyroid hormone and estrogen on a fragment of the hPRL promoter containing both the proximal promoter and the distal regulatory region (p2627PRLCAT; Fig. 6). The results

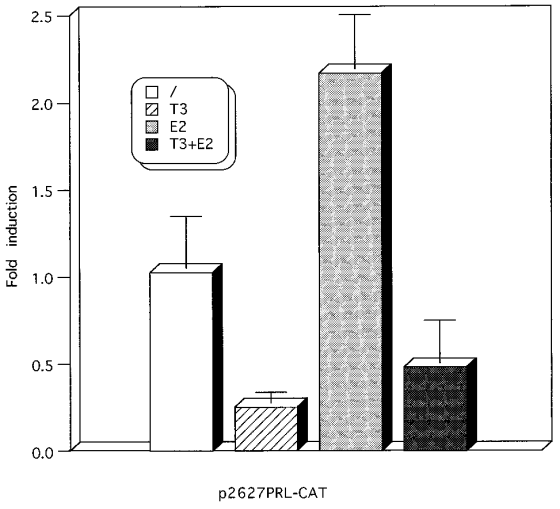

Fig. 6. The Inhibitory Effect of T_3 Overrides the Stimulatory Effect of E_2 Exerted via the 2627-bp hPRL Promoter Fragment

GH_3B_6 cells were electroporated with 30 μg p2627PRLCAT. After electroporation, the cells were incubated for 48 h in the presence of the antiestrogen ICI 64384 (1 μM) and/or T_3 (10 nM) or in the presence of E_2 (100 nM) and/or T_3 (10 nM). CAT activity was measured, and the induction factors were calculated as the ratio between the measured activity to the value obtained with ICI-treated control cells. The data are the mean ± SEM of five independent experiments, each performed in duplicate.

show, as expected, that T_3 exerts an approximately 3-fold inhibitory effect, and E_2 has a 2-fold stimulatory effect. In cells treated with both hormones, the recorded CAT activity is surprisingly low, reaching only half the basal level. In conclusion, the impact of the distal element on T_3 regulation appears to be weak, even when it is stimulated by estrogens.

DISCUSSION

We have studied, for the first time, the regulatory effects of thyroid hormone on the hPRL gene promoter. We present evidence that thyroid hormone down-regulates transcription of the hPRL gene in pituitary cells. In the hPRL promoter we have identified two main T_3-responsive regions. The first, located in the proximal promoter, mediates a strong negative effect, whereas the second, located in the distal promoter, mediates a weak positive response. The overall effect of the two combined regions is repressive.

Our finding that T_3 inhibits the hPRL promoter is in keeping with the physiological data on the endogenous human gene. Hypothyroidism in human patients is frequently associated with increased serum PRL levels, whereas hyperthyroid patients present lower PRL levels than euthyroid control subjects (40). Although it is unclear whether this effect is due to direct regulation of the hPRL gene, recent studies on a human pituitary cell line suggest 2-fold inhibition of endogenous hPRL gene transcription by T_3 (41).

The Proximal Promoter

The proximal hPRL promoter mediates T_3 inhibition of a heterologous promoter (TK) in both pituitary (GH_3B_6) and nonpituitary cells (HeLa, CV1, and JEG-3; Fig. 2). On the other hand, we observe no binding of TR to the proximal T_3 response element in gel retardation assays (Fig. 3). This suggests that T_3 acts indirectly. Recently, T_3 treatment was shown to inhibit Pit-1 gene transcription through interference between the thyroid hormone receptor and Pit-1 (42). As T_3 inhibition of the hPRL promoter is observed in nonpituitary cells (Fig. 2c), we can exclude an indirect action of T_3 through inhibition of Pit-1 expression. Furthermore, coexpression of Pit-1 and TR does not affect T_3 inhibition in HeLa cells (Fig. 2c), so we can rule out a mechanism involving interference between the TR and Pit-1 leading to inhibition of the hPRL gene promoter, nor is the forskolin pathway involved in the response of the hPRL promoter to T_3 (our data not shown). This contrasts with T_3 inhibition of Pit-1 gene expression, which involves interference of T_3 with the cAMP induction pathway (42).

An AP-1-responsive element has been located in the hPRL proximal promoter between coordinates −61 and −54 (see also Caccavelli, L., I. Manfroid, J. A. Martial, and M. Muller, in preparation). It contains an AP-1-binding sequence that differs by only one mismatch from the consensus AP-1-binding site. We show that this sequence can specifically bind AP-1 in gel retardation assays (Fig. 5) and that it mediates 15-fold stimulation of reporter gene transcription in the presence of coexpressed c-*jun* and c-*fos* (Fig. 4b). This stimulation is almost completely abolished in the presence of hormone-bound TR. We conclude that thyroid hormone exerts its inhibitory effect via interaction of hormone-bound TR with AP-1. Furthermore, mutation of the AP-1 site completely abolishes the stimulatory effect of c-*jun* and c-*fos* and the inhibitory effect of T_3 (Fig. 4). This confirms that the two responses are interrelated.

The transcription-stimulating activity of AP-1 is increased by protein kinase C phosphorylation. Gellersen *et al.* (34) observed synergistic stimulation of the hPRL proximal promoter (250 bp) by TPA (12-*O*-tetradecanoyl-phorbol-13-acetate) treatment and Pit-1 expression in the SKUT-1B-20 human uterine cell line. Here we show strong stimulation of the hPRL promoter in HeLa cells cotransfected with a reporter construct and with c-*jun* and c-*fos* expression vectors, but this effect is completely Pit-1 independent. This discrepancy suggests that TPA stimulation in SKUT 1B-20 cells might involve another mechanism(s) in addition to the increased AP-1 activity.

Several groups have reported interaction of AP-1 with members of the nuclear receptor family, *e.g.* the glucocorticoid, retinoic acid, progestin, estrogen, and

thyroid hormone receptors (15–22). Different mechanisms have been observed for this cross-modulatory action according to the promoter context (22). Direct interaction between TR and AP-1 has been demonstrated in coimmunoprecipitation assays (20). Furthermore, in gel retardation experiments, binding of AP-1 to DNA was inhibited by TR when the latter was added before addition of the labeled binding sequence (19, 20). The hPRL proximal promoter contains one AP-1-responsive element and no TR-binding site. This suggests that TR blocks the action of AP-1 by hindering its binding to its response element. Whether the interaction between TR and AP-1 is direct or mediated by another factor(s) is unclear.

In striking contrast to the overall inhibition of hPRL transcription by T_3, the combined positive and negative acting elements of the rPRL promoter mediate overall activation of this promoter by T_3 in GH_3 cells (39). The rPRL promoter responds differently to T_3 according to the pituitary cell line examined (36–39). Nevertheless, studies in rats have shown that treatment with the antiarhythmic drug amiodarone, a T_3 antagonist, reduces PRL mRNA levels (43), suggesting that rPRL expression is up-regulated by T_3. The rat sequence corresponding to the hPRL proximal AP-1 sequence differs by three nucleotides from the AP-1 consensus binding sequence, suggesting that rat and human PRL genes might be differently regulated by AP-1. Indeed, recently it was shown that expression of c-*jun* inhibits rat PRL expression in GH_4 pituitary cells (44). This repression involves the FPII region centered at −125 in the rat promoter and is only seen in GH_4 cells. The proposed mechanism is an indirect binding of c-*jun* to the promoter by recruitment of a pituitary-specific FPII-binding factor. Whether T_3 would be able to block this repression, which would result in an overall activation by T_3, is at present unknown. These observations contrast with our results using the hPRL promoter in GH_3B_6 cells. However, the same researchers describe a synergistic activation of the rPRL promoter by c-*jun* and Pit-1 expression in HeLa cells. In this case, the proposed mechanism is an interaction of c-*jun* with Pit-1 bound to the proximal binding site, but again without direct DNA binding of c-*jun*. Nothing is known about T_3 modulation of this effect, which is suppressed in GH_4 cells by the c-*jun* repression described above. Thus, it seems very likely that the mechanism of T_3 inhibition of the hPRL promoter described here does not apply to the homologous rPRL promoter.

Complete hPRL Promoter

The combined action of the proximal, negative promoter and the distal, weakly positive region results in inhibition by T_3 of the complete upstream region. In addition, this region contains a consensus estrogen-responsive element (ERE) located close to a TR-binding sequence (see also Van de Weerdt, C., F. M. Pernasetti, L. Caccavelli, J. A. Martial, and M. Muller, in preparation). The same hPRL ERE can mediate 2-fold stimulation of a heterologous promoter in SKUT-1B-20 cells, a PRL-producing uterine carcinoma cell line (34). The fact that T_3 is also able to block E_2 activation of this construct (Fig. 6) stresses the importance of the T_3 regulation mediated by the proximal AP-1 site.

Activation of rPRL gene transcription by E_2 involves the stabilization of a chromatin loop permitting critical interactions between proteins of the distal enhancer and proximal promoter (45). Dexamethasone-bound GR has been found to bind to the distal ERE, competing in this with estrogen receptor and preventing formation of the loop (46). We have no information as to whether a particular chromatin structure characterizes the plasmid constructs used here, but hindering of loop formation by TR is certainly a possible mechanism of repression, especially as factors bound to the proximal promoter are known to be important in loop formation. If the AP-1 complex plays a role in the interactions between the distal and proximal regions, then T_3 blocking of AP-1 binding to the DNA would inhibit loop formation, thus preventing E_2 stimulation. Alternatively, T_3 might down-regulate the expression of other proteins necessary for the response to estrogen mediated by the complete hPRL promoter.

In conclusion, we demonstrate that hPRL gene transcription is inhibited by thyroid hormone. We show that the inhibitory effect of T_3 involves interference with the AP-1 transactivation mediated by an AP-1-binding site located in the proximal hPRL promoter. A weak positive TRE, located in the distal region close to an ERE, modulates this inhibition. However, T_3 treatment reduces the E_2 stimulatory effect at a promoter region containing both proximal and distal response elements. Thus, our work illustrates the complexity of mechanisms involved in gene transcriptional regulation. Cross-talk between nuclear receptors and other transcription factors provides an intricate set of distinct regulatory mechanisms permitting precise control of a specific gene expression by intra- and extracellular factors.

MATERIALS AND METHODS

Plasmid Constructs

PRLCAT constructs with 5′-deletions in the hPRL promoter (−4777, −3474, −2627, −1750, −1330, −740, and −250 indicate the first remaining upstream nucleotide) have been described (30, 32). The chimeric hPRLTKCAT construct p(−250/−35)TKCAT was constructed as follows. The DNA fragment was amplified by PCR using strategically designed 5′- and 3′-primers (see oligonucleotides), digested by *Hin*dIII and *Xba*I, gel purified, and inserted in front of the TK promoter by ligation into the *Hin*dIII-*Xba*I-digested pBLCAT2 (47) plasmid. Plasmid p164PRL-LUC was built by digesting p164PRLCAT (32) with *Bgl*II and *Hin*dIII, after which the hPRL promoter fragment was gel purified and cloned into the pXP2 vector (48). Plasmid p164(AP-1)mPRL-LUC was built using the Chameleon double stranded, site-directed mutagenesis

kit from Stratagene (La Jolla, CA), using the mutated AP-1 m oligonucleotide as a template. The sequence of all constructs was verified by dideoxy chain termination sequencing. The (TREpal)3XTKCAT construct has been described previously (14). The expression vector pSV2c-*erb*Aα (containing human TRα cDNA expressed under the control of the simian virus 40 early promoter) was provided by J. Ghysdael. The pRSVc-*jun* and pRSVc-*fos* vectors, which contain coding sequences for c-*jun* and c-*fos* under the control of the Rous sarcoma virus (RSV) promoter, respectively, are gifts from P. Herrlich. The pRSVβgal and the pRSVPit-1 expression vectors have been described previously (49, 50). All plasmids were prepared using the Nucleobond kit from Macherey-Nagel Co. (Düren, Germany).

Oligonucleotides

The double or single stranded oligodeoxyribonucleotides used for plasmid construction or gel mobility assays were obtained from Eurogentec (Seraing, Belgium). The AP-1 oligonucleotide contains the AP-1 site of the human collagenase promoter (56). The numbers represent positions in the PRL upstream sequence, where relevant.

For each oligonucleotide the sequence of one strand is presented: −250PRL, 5′-CCCAAGCTTAGATCTCACCTTTCAAC-3′; −35PRL, 5′-GCTCTAGATATCTTCATGAATATAATG-3′; DR4, 5′-TCGAAGCTTCAGGTCACAGGAGGTCAAGCT-3′; AP-1 m, 5′-CTTCATGAATATAATCGAGCAGGCATTCGTTTCCC-3′; and Sp1, 5′-AGTTCCGCCCATTCTCCGCCCCA-3′.

Cell Cultures and Transient Transfection

GH_3B_6 and HeLa cells were grown in monolayers in DMEM supplemented with 10% FCS plus E_2 (5×10^{-10} M). Twenty-four hours before transfection, the cells were grown in phenol red-free DMEM supplemented with 2% AG1X8 resin-charcoal-stripped FCS (52). T_3, E_2, and forskolin were obtained from Sigma (Deisenhofen, Germany).

Electroporation GH_3B_6 cells were harvested with trypsin-EDTA and resuspended in phenol red-free DMEM with 2% depleted FCS (FCSt; final concentration, 2×10^6 cells/ml). Thirty micrograms of each plasmid were mixed with 1.6×10^6 cells. The cells were then exposed to a single pulse of 250 V/4 mm and 1500 μF capacitance with an Easyject electroporator (EquiBio, Seraing, Belgium). Transfected cells were immediately transferred to phenol red-free DMEM with 2% FCSt and incubated for 48 h. Treatment of the electroporated cells is detailed in the figure legends.

Calcium Phosphate Precipitation HeLa cells were transfected by the $CaPO_4$ method, as previously described (53). The day before transfection, 5×10^5 cells were plated in petri dishes (9 cm in diameter) in phenol red-free DMEM with 2% FCSt. Two to 10 μg reporter plasmid were mixed with 5 μg of each expression vector specified; the total amount of expression vector was adjusted to 20 μg by the addition of the appropriate amount of pRSVβgal. After a 12-h incubation with $CaPO_4$ precipitate, the cells were washed with PBS and incubated in phenol red-free DMEM with 2% FCSt for 48 h. Hormone treatments are detailed in the figure legends.

CAT Assay After a 48-h incubation, the cells were harvested by scraping and resuspended in 100 μl 250 mM Tris-HCl (pH 7.6). Cell disruption and the CAT assay were performed as described previously (32).

Luciferase Assay After a 48-h incubation, the cells were harvested by scraping and directly resuspended in lysis buffer. The assays were performed as described previously (54).

Protein concentrations in extracts were determined by the Bradford assay. Fifty micrograms of total extract were used in both the CAT and LUC assays.

Mobility Shift Assays

Oligonucleotides and PCR fragments were [^{32}P]ATP labeled using T4 polynucleotide kinase. Labeled probes were purified by elution from polyacrylamide gels. Proteins were preincubated in a buffer containing 20 mM HEPES (pH 7.8), 4 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 10% glycerol, 0.2 mg/ml BSA, and 80 mM KCl with 1 μg poly(dI-dC) for 30 min at 4 C. When necessary, antibodies were preincubated with proteins for 30 min. After preincubation, 10,000 cpm probe were added, and incubation proceeded for 30 min at 4 C. The resulting protein-DNA complexes were resolved by electrophoresis on a prerun 5% polyacrylamide gel with 0.5 × TBE as the running buffer for 2 h at 4 C. The gel was dried and autoradiographed overnight. Polyclonal rabbit anti-Jun antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antigen sequence used to produce the antibodies corresponds to residues 247–263 within the C-terminal DNA-binding domain of the mouse c-Jun protein. Polyclonal rabbit antithyroid hormone receptor α1 antibody was purchased from Affinity Bioreagents (Neshanic Station, NJ). The antigen sequence used to produce the antibodies corresponds to residues 403–410 of the extreme C-terminal region of the human TRα, which is identical to the rat sequence. Recombinant RXR was provided by Dr. Baes, University of Leuven (Leuven, Belgium).

Bacterial Expression and Purification of hTRα1 The bacterial strain BL21(DE3)pLYS S (55) was transformed with the vector pET-hTRα1 (provided by L. J. De Groot). A freshly transformed clone was used to inoculate 500 ml Luria Bertoni medium. The culture was grown at 37 C to an OD of 0.4, isopropyl-β-D-thiogalactoside (IPTG) was added to a 0.5-mM final concentration, and the culture was gently rocked for 5 h at 20 C. The bacteria were harvested by centrifugation and resuspended in 20 ml lysis buffer (20 mM Tris, pH 8.0; 100 mM NaCl; 0.5 mM $MgCl_2$; 1 mM phenylmethylsulfonylfluoride; and 1% aprotinin). The suspension was sonicated on ice to obtain a clear lysate; 2 ml of a mixture of 0.1 M DTT, 0.5 M EDTA, and 5% Nonidet P-40 were added; and bacterial debris were removed by centrifugation at 60,000 × *g*. Soluble proteins were precipitated from the supernatant by the addition of 0.33 g/ml ammonium sulfate and recovered by centrifugation at 60,000 × *g*. The pellet was resuspended in 1 ml HS buffer (25 mM HEPES-KOH, pH 7.6; 5 mM $MgCl_2$; 1 mM EGTA; 1 mM DTT; and 10% glycerol), and the solution was dialyzed against HS buffer. The fraction was loaded on a heparin-Sepharose column, and proteins were eluted with a linear KCl gradient. Human TRα1 was detected in the fractions by means of a gel retardation assay using a DR-4 probe and by SDS-PAGE followed by Coomassie staining or Western blotting. Eluates at 0.4–0.7 M KCl contained greater than 95% pure, soluble, bacterially expressed hTRα1. We used 4 μg purified TR extract in each gel retardation assay unless otherwise indicated in the figure legends.

HeLa Cell Extracts Enriched with AP-1 Complex HeLa cells were transfected with 25 μg pRSVc-*jun* and pRSVc-*fos* according to the diethylaminoethyl-dextran transfection protocol (56). The cells were harvested 48 h after transfection. Total protein extracts were prepared by resuspending the cells in 20 mM HEPES, pH 7.8; 400 mM KCl; 20% glycerol; and 2 mM DTT. They were disrupted by three freeze-thaw cycles, followed by centrifugation at 12,000 rpm for 30 min. Total enriched extract was added in each gel retardation assay in an amount corresponding to 4.5 μg protein, as determined by the Bradford assay.

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