Transcriptional Regulation by Triiodothyronine Requires Synergistic Action of the Thyroid Receptor with Another trans-Acting Factor

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Human placental lactogen B (hCS-B) promoter activity is strongly stimulated by triiodothyronine (T3) in pituitary GC cells through interaction between the thyroid receptor and a thyroid receptor-binding element (TBE) spanning coordinates -67 to -41. This TBE is adjacent to the binding site for pituitary factor GHF1 (-95 to -68) which seems necessary for T3 stimulation of hCS-B promoter activity (M. L. Voz, B. Peers, A. Belayew, and J. A. Martial, J. Biol. Chem. 266:13397-13404, 1991). We here demonstrate actual synergy between the thyroid receptor and GHF1. Indeed, in pituitary JEG-3 cells devoid of factor GHF1, hCS promoter activity is barely stimulated by T3, while a strong response is observed in pituitary GC cells. In the latter, furthermore, neither the TBE nor the GHF1-binding site alone is sufficient to render the thymidine kinase promoter responsive to T3, while in combination they promote strong T3 stimulation. Close proximity between these sites is required for optimal synergy: T3 stimulation globally decreases with increased spacing. Furthermore, synergy occurs not only with a GHF1-binding site but also with all other factor recognition sequences tested (Sp1, NF1, C1, Oct1, and CACCC boxes) and even with two other copies of the TBE. Nor is it specific to hCS TBE, since the palindromic sequence TCAGGTCA TGACCTGAA (TREpal) also exhibits cooperativity.

In vertebrates, thyroid hormones critically influence development, mainly by regulating the transcription of specific genes. These hormones interact specifically with nuclear receptors that regulate transcription of target genes by binding to specific DNA sequences called thyroid hormone response elements (TREs) (reviewed in reference 22). Most TREs described to date are composed of two or more copies of the motif GGCTA (or a related sequence), arranged as direct or inverted repeats (12-32). For example, a palindromic arrangement of this core motif, GGCTA TGACC, confers transcriptional stimulation by T3 (10), as does a direct repeat spaced with 5 bp: GGCTA(N)GGCTA (32, 33). In contrast, this same direct repeat spaced with only 3 bp can permit negative regulation by the thyroid hormones (19).

We have previously demonstrated strong stimulation of human placental lactogen B (hCS-B) promoter activity by thyroid hormones in rat pituitary GC cells (34). This requires the presence of a thyroid receptor-binding element (TBE) spanning coordinates -67 to -41 as shown by DNase I footprinting. The TBE sequence is not sufficient by itself to confer T3 stimulation, and that is why we are favoring the use of TBE versus TRE for this sequence. Interestingly, this TBE sequence (GGTGCGGTCAAGCAGGAGGAGGA) does not contain any obvious repeats or palindromes but is composed of two different domains, one containing the motif GGCTA and the other being very rich in purines. In the hCS-B promoter, the TBE is adjacent to a sequence binding the pituitary-specific factor GHF1, also named Pit-1 (coordinates -95 to -68). Surprisingly, this latter sequence seems also necessary to allow T3 stimulation of hCS promoter activity, as its deletion abolishes the T3 response. This has led us to define a thyroid response unit including both the TBE and the GHF1-binding site and to hypothesize that the GHF1 factor and thyroid receptor (TR) act cooperatively.

We here confirm the above hypothesis and show that many other factors can act synergistically with the TR. We further show that the TREpal sequence (10) also allows this synergy.

MATERIALS AND METHODS

Plasmid construction. Plasmid CS493CAT has been previously described (34). The TBE-TKCAT, TBE-CACCC-TKCAT, TBE-CPI-TKCAT, TBE-Sp1-TKCAT, TBE-Oct1-TKCAT, and TBE-NF1-TKCAT constructs were respectively derived from plasmids pG29C*tkCAT, pG29C tkCAT, pG29CpttkCAT, pG29SPtkCAT, pG29OTtkCAT, and pG29NFtkCAT (26), kindly provided by R. Renkawitz's laboratory, by replacing the glucocorticoid response element (HindIII and SmaI digestions) with the hCS TBE (-67 to -41) (see Fig. 3A for sequences). The TBE23GHF1-TKCAT construct was derived from the TBE-CACCC-TKCAT construct by replacing the CACCC protein-binding site (SaltI and BamHI digestions) with the GHF1-binding site of the hCS promoter (-94 to -71). It thus contains a 26-bp linker separating the two sites. All other distance mutants were obtained by appropriate digestion(s), Klenow treatment, and ligation (see Fig. 2A for sequences). TREpal-TKCAT, TREpal3GHF1-TKCAT, and TREpal-NF1-TKCAT were respectively obtained from TBE-TKCAT, TBE6GHF1-TKCAT, and TBE-NF1-TKCAT by replacing the TBE (HindIII and SmaI digestions followed by Klenow treatment of these constructs) with TREpal (gatcTCAAGTCAATGACCTGAA gate) (10). GHF1-TKCAT was produced by removing the TBE of the TBE6GHF1-TKCAT construct, and TKCAT
was produced by deleting the TBE from TBE-TKCAT, (TBE)\textsubscript{3}-TKCAT and (TREpal)\textsubscript{3}-TKCAT were constructed by replacing the glucocorticoid response element of pSV2\textsubscript{C}tCAT by three copies of TBE or TREpal, respectively. The structures of all mutants were confirmed by sequencing.

To avoid artificial synergistic effects, the fortuitous NF1-binding site created at the junction of the polylinker and pUC sequences was deleted from all constructs by removing a 215-bp NdeI-HindIII fragment (27).

J. Ghyssael kindly provided the pSV2-cerbA\textsubscript{ox} expression vector containing human TR\textsubscript{α} cDNA expressed under the control of the simian virus 40 early promoter.

**Cell culture conditions.** GC cells were grown in Ham’s F-12 medium supplemented with 15% fetal calf serum (FCS), and the JEG-3 cells were grown in minimal essential medium supplemented with 10% FCS. One day before transfection, the cells received phenol-red-free medium supplemented with FCS treated with AG1-X8 resin (23) and with activated charcoal to eliminate thyroid and steroid hormones (FCS).

**Transfection by electroporation.** GC and JEG-3 cells were harvested with trypsin-EDTA and resuspended in phenol-red-free Ham’s F-12 medium–15% FCS, at a concentration of $3 \times 10^6$ cells per ml for GC cells and in phenol-red-free minimal essential medium supplemented with 10% FCS, at a concentration of $15 \times 10^6$ cells per ml for JEG-3 cells. Purified plasmid DNA was mixed with 800 μl of cell suspension and exposed to a single pulse delivered by a Cellject electroporator (EquiBio, Liège, Belgium). For GC cells the pulse parameters were 275 V/4 mm and a 1,500-μF capacitance; for JEG-3 cells the values were 200 V/4 mm and 1,050 μF. Cells were rapidly transferred to phenol-red-free medium supplemented with FCS. Half of the transfected cells were incubated with 10 nM T3, and the other half received the amount of ethanol-NaOH used to dissolve the T3. Forty hours after electroporation, the cells were harvested by scraping, washed in phosphate-buffered saline, resuspended in 100 μl of 250 mM Tris-HCl (pH 7.6), and frozen at −70°C. GC cells were disrupted by three cycles of freezing and thawing, and JEG-3 cells were disrupted by four cycles of freezing and thawing with sonication. The samples were centrifuged at 12,000 × g in an Eppendorf centrifuge, and chloramphenicol acetyltransferase (CAT) assays were performed as described by Seed and Sheen (28). The T3 induction ratio was expressed as the ratio of CAT activities in T3-treated versus untreated cells.

**Statistical analysis.** Means were statistically compared by two-way variance analysis.

**RESULTS**

(i) hCS-B promoter activity is stimulated by T3 in pituitary GC cells but not in placental JEG-3 cells. To investigate whether the pituitary GHF1 factor is absolutely necessary for T3 induction of hCS promoter activity, we compared this promoter’s T3 regulation in cells possessing and devoid of this trans-acting factor (pituitary GC cells and placental JEG-3 cells, respectively). As JEG-3 cells lack a functional TR, we cotransfected them with an expression vector encoding the human TR$\alpha$ (pSV2-cerbA\textsubscript{ox}). A preliminary dose-response study showed an optimal T3 response for 10 μg of expression vector (data not shown). Table 1 shows for GC and JEG-3 cells the T3 response of CS493CAT, which contains the 493-bp hCS promoter upstream of the CAT gene. The response is much weaker in the JEG-3 cells (2-fold) than in GC cells (10-fold). This is not due to an inability of JEG-3 cells to respond to T3 treatment, since stimulation of the positive control (TREpal)$_{3}$-TKCAT is strong in this cell line (20-fold). The latter construct consists of three copies of TREpal (10) upstream from the herpes simplex virus thymidine kinase (TK) promoter fused to the CAT gene. Thus, while CS493CAT is stimulated as strongly as (TREpal)$_{3}$-TKCAT in GC cells, its stimulation is 10-fold weaker in JEG-3 cells. These experiments corroborate the view that the presence of GHF1 is crucial to obtaining optimal T3 stimulation of hCS promoter activity.

(ii) Cooperativity between the TR and the GHF1 factor. To see whether the TR and the GHF1 factor act cooperatively, the corresponding binding sites were cloned independently or in combination upstream from the hybrid TKCAT gene and the resulting constructs were tested for their response to T3 in transfected GC cells. Plasmid TBE-TKCAT, containing only the hCS TBE 72 bp upstream from the TK promoter, presented a 2.0-fold stimulation, slightly above the 1.9-fold nonspecific stimulation observed with the TK promoter alone (Fig. 1). Similarly, the GHF1-binding site of the hCS promoter was unable to confer specific stimulation (1.3-fold) to the TK promoter. When both binding sites were combined (TBE&GHF1-TKCAT), stimulation was strong (5.5-fold), the effect being significantly greater than that of the TBE or the GHF1-binding site alone (P < 0.001). This clearly demonstrates synergy between the TR and GHF1.

(iii) Effect of spacing between the two binding sites on TR-GHF1 synergy. In the TBE&GHF1-TKCAT construct described above, the TBE and the GHF1-binding site were juxtaposed in the same relative orientation and with the same spacing as naturally found in the hCS promoter. To determine whether synergy could be altered by increasing the distance between the two binding sites, we generated various constructs containing 0 to 31 additional base pairs (Fig. 2A). Figure 2B shows the T3 induction ratio to vary with spacing: cooperativity is strongest for the natural relative position (0-bp insert) and with the 10-bp insert. As the distance further increases, the general tendency is a weakening of T3 induction, which finally drops to nearly the level obtained with the TBE alone: 2.6- and 2.8-fold induction with 27- and 31-bp inserts, respectively, compared with 2.1-fold with the TBE alone.

(b) Many transcription factors can act synergistically with the TR. To determine whether trans-acting factors other than GHF1 might act synergistically with the TR, the TBE was cloned in combination with different ubiquitous-factor-binding sites: the CACCC box found in the tryptophan oxygenase promoter (8), the CCAAT box of the long terminal repeats of the murine sarcoma virus, recognized by CP1 (6), the GC boxes I and II of the simian virus 40 enhancer

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**TABLE 1.** hCS-B promoter activity stimulation by T3 in pituitary GC cells and placental JEG-3 cells

<table>
<thead>
<tr>
<th>Construct</th>
<th>T3 induction ratio in:</th>
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<tr>
<td></td>
<td>JEG-3 cells</td>
</tr>
<tr>
<td>CS493CAT</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>(TREpal)$_{3}$-TKCAT</td>
<td>20 ± 2.8</td>
</tr>
<tr>
<td>TKCAT</td>
<td>0.8 ± 0.1</td>
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* GC cells were transfected with 30 μg of CS493CAT or 10 μg of (TREpal)$_{3}$-TKCAT or TKCAT, and JEG-3 cells were transfected with 50 μg of these three constructs plus 10 μg of pSV2-cerbA\textsubscript{ox}. The transfected cells were treated with T3 as described in Materials and Methods. T3 induction ratios are means ± standard errors from at least two independent transfection experiments performed in triplicate.
which bind Spl (2), the octamer sequence found in the immunoglobulin heavy chain enhancer and recognized by the ubiquitous factor Oct1 (21), and the NFI-binding site consensus sequence (6, 16) (Fig. 3A).

Figure 3B shows that stimulation is significantly increased by combining the TBE with any of these factor-binding sites ($P < 0.001$). The increase is at least equivalent to that produced by the GHFI-binding site at the same position. The NFI-binding site produces an even stronger synergistic response than does the GHFI site. Strong stimulation was also observed when three copies of the TBE were inserted head to tail upstream from the TK promoter. This shows that the TR acts cooperatively with every tested transcription factor and even with other TRs bound to adjacent sites.

(v) **Cooperativity is also observed with a palindromic sequence.** As the hCS TBE contains no direct or inverted repeats as generally found in consensus TRE sequences, we wondered whether the observed synergy might be specific to the hCS TBE. We therefore tested another type of TR-binding sequence, TREpal: TCAAGTCA TGACCTGA (10). Like the TBE, TREpal was inserted 72 bp upstream from the TK promoter in one or three copies and also in combination with either the GHFI- or NFI-binding site. The effect is the same as with hCS TBE (Fig. 4): induction is weak with TREpal alone (2.8-fold compared with the 1.9-fold nonspecific induction of TKCAT), but when the TREpal is combined with the GHFI- or NFI-binding site or with two other TREpal sequences, it produces a strong T3 response. Inducibility of the TREpal, like that of the TBE, is thus greatly increased by juxtaposition with another transcription factor-binding site.

**DISCUSSION**

We have previously shown strong stimulation of hCS promoter activity by T3 in pituitary GC cells, 5' and 3' deletion studies indicate that the minimal DNA sequence required to produce this stimulation spans coordinates -97 to -31 in the hCS promoter. Interestingly, this thyroid response unit contains not only a TBE (-67 to -41) but also a GHFI-binding site (-95 to -68), suggesting cooperation between the TR and the GHFI factor (34).

The present study confirms the above hypothesis. Indeed, the weak T3 induction of hCS observed in JEG-3 cells lacking the GHFI factor suggests that GHFI is indeed crucial to obtaining a full T3 response. In GC cells, neither the TBE nor the GHFI-binding site is able to confer a response to T3 when inserted alone upstream from the TK promoter, while together they promote strong T3 stimulation. Furthermore, synergy is optimal when the sites are close together, globally decreasing with increased spacing. Finally, this synergy is specific neither to the hCS TBE sequence nor to the GHFI-binding site, as it is also observed with the TREpal sequence (10) and with all ubiquitous-factor-binding sites tested.

Our study thus points to a functional interdependence of adjacent cis-acting elements, one bound by the TR (as a homo- or heterodimer; see below) and the other bound by transcription factors. To our knowledge, this is the first demonstration of such cooperativity with the TR, although other studies have pointed in this direction: Ye et al. showed that both cell-specific elements and TREs are required for efficient T3-regulated expression of the rat growth hormone gene (36). Likewise, myosin heavy chain genes can be regulated by thyroid hormones in opposite directions, depending on the tissue in which they are expressed (11, 31). These data point to the existence of cooperative interactions between TR and other factors. While this paper was submitted, a study of the proximal rat growth hormone promoter region indicating a synergistic activation by the TR and GHFI was published (24), thus confirming with a homologous promoter the data presented here.

For steroid receptors, on the other hand, such functional interactions are well documented. Synergy has been observed between two adjacent receptor-binding sites (1, 5, 12, 26) and between a receptor-binding site and an adjacent transcription factor-binding site (4, 9, 26, 27, 29). Such synergy might be achieved by cooperative binding to DNA (4, 15, 25, 30, 35) or by some unknown mechanism unrelated to DNA binding (14, 18, 20).

![FIG. 1. Synergy between the TR and GHFI. T3 induction ratios were observed after transfection of GC cells with chimeric TKCAT constructs (10 or 30 μg) and treatment with T3 as described in Materials and Methods. Values are means ± standard errors from at least two independent transfections performed in triplicate. The T3 induction ratio obtained for CS493CAT in these experiments is 9 ± 1.4.](http://mcb.asm.org/)
FIG. 2. Effect of spacing between the TBE and the GHF1 box. (A) Sequences of the different TBE-GHF1-TKCAT spacing mutants (n represents the distance artificially introduced between the TBE and the GHF1-binding site). (B) T3 induction ratios observed after transfection of GC cells with TBE-GHF1-TKCAT spacing mutants (5 or 10 μg) and treatment with T3 as described in Materials and Methods. Values are means ± standard errors from at least two independent transfections performed in triplicate. The T3 induction ratio obtained in these experiments for the TBE-TKCAT is represented by an arrow (2.1-fold).

It is quite surprising that synergy occurs with all transcription factors tested, as these are unrelated and each possesses different DNA-binding and transactivating domains. This points to a general system of interactions between proteins that regulate transcription and the TR. The glucocorticoid receptor likewise acts in concert with a whole battery of trans-acting factors (4, 26, 27, 29). Multiple domains of the glucocorticoid receptor were found to be involved in synergistic activity (17).

The fact that synergy appears with both the TRs and the steroid receptors suggests that this phenomenon is common to all members of the thyroid-steroid receptor superfamily, for example, retinoic acid receptors (RARs), retinoid X receptors (RXRs), and vitamin D receptor (VDR), for which such a property has not been described yet.

Gradual spacing of the TBE and the GHF1-binding site does not reveal any clear 10-bp periodicity of the synergistic response as observed with some factors (20, 27). This lack of
FIG. 3. Many transcription factors can act synergistically with the TR. (A) Schematic representation of the different combinations of the TBE with transcription factor-binding sites. (B) T3 induction ratios observed after transfection of GC cells with these chimeric TKCAT constructs (10 or 30 μg) and treatment with T3 as described in Materials and Methods. Values are means ± standard errors from at least two independent transfections performed in triplicate.
with TREpal, with similar results. This suggests that any TR-binding sequence might exhibit this synergy. It would be interesting to check, however, whether a perfect direct repeat behaves in the same way. Our result that one copy of the TREpal sequence does not confer responsiveness to T3 is apparently in disagreement with other studies (7, 10). However, detailed examination of these other reports shows that the TREpal sequence was cloned very near trans-acting factor-binding sites in the vectors used by these authors: Glass et al. cloned the TREpal sequence directly upstream from the 107-bp TK promoter and thus less than 10 bp upstream from an Sp1-binding site (10). In the construct of Damm et al. (7), the TREpal sequence was inserted into the HindIII site of pBLCAT2, i.e., directly adjacent to the cryptic NF1-binding site artificially created by junction of the polynucleotide and pUC sequences (27). In our case, the TREpal sequence was cloned in an environment devoid of any nearby trans-acting factor-binding site, since we linked it to the TK promoter by a 72-bp linker and we deleted the fortuitous NF1-binding site. This different environment is probably responsible for the different regulatory behavior observed.

Our results thus underline the importance of the cis elements flanking the TBE and the need to take neighboring sequences into account when investigating such TBEs.

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