Characteristics of a Negative Thyroid Hormone Response Element*

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Thyroid hormones may stimulate or repress transcriptional activity depending upon the specific gene. Whereas, a palindromic DNA sequence, TREpal, mediates positive regulation by thyroid hormone, the negative response element (nT3RE) remains undefined. Therefore, we have examined the DNA sequences that mediate the inhibitory effects of thyroid hormone on the transcription of the β-subunit gene of rat thyrotropin (rTSHβ). In rat pituitary tumor cells (GH4), transient expression of plasmid constructs containing the putative nT3RE of rTSHβ mediated negative regulation by L-triiodothyronine (T3). Since this nT3RE contained sequences which resembled a half-site motif of the consensus T3RE and the idealized palindromic (TREpal), we tested a construct containing this half-site motif in the same cells. T3 decreased the activity of this plasmid. Co-transfection studies in T3 receptor (T3R)-deficient cells indicated that either α or β isoforms of T3R were required for the inhibitory effects of the hormone. Both T3R isoforms bind to DNA sequences containing the nT3RE from rTSHβ DNA or the half-site motif of TREpal. In summary, our results show that the repressive properties of T3 are mediated by a nT3RE from rTSHβ. Unexpectedly, this motif resembles a half-site component of TREpal which enhances promoter activity in response to T3.

Thyroid hormone regulation of gene expression includes both strong inductive as well as repressive effects (1, 2). A consensus palindromic derived from the rat growth hormone (rGH)3 gene mediates positive regulation by T3 (3–5). Although the inductive effects of T3 are well characterized, the repressive effects are not. We have shown recently that the repressive effects of T3 on rat TSHβ subunit gene transcription is mediated through a 17-base pair DNA fragment (CCG-CAGTGCAGAATGAC; negative effect being independent of the fragment position relative to the transcriptional start site (6)). Within this negative T3 response element (nT3RE), there is a 6-base pair motif (AAGTAA) which shares homology with a portion of the rGH T3RE referred to as site “A” (AGGTAA). When the A site is positioned adjacent to an imperfect inverted repeat (TAGGACGGACACC) referred to as sites “B” and “C,” respectively, this element mediates the positive effects of T3 on rGH gene transcription (4).

Sites A, B, and C are considered to be “half-sites” of a positive T3RE. This observation gave rise to the notion that positive T3REs, such as found in the rGH gene and TREpal, are comprised of more than on repeat of a half-site, whereas a nT3RE is formed by a single copy of the half-site motif. In support of this hypothesis, recent studies of other genes which are negatively regulated by T3 such as thyrotropin-releasing hormone (7), epidermal growth factor receptor (8), as well as the α-subunit of rTSH (9, 10) have revealed 6-base pair motifs that resemble the nT3RE of rTSHβ. Therefore, we have examined the possibility that differential regulation by T3 may be attributed to the presence of a single or multiple repeats of a core motif by measuring the ability of a nT3RE from rTSHβ and the half-site motif of TREpal to confer negative regulation to the heterologous thymidine kinase (TK) promoter.

MATERIALS AND METHODS

Construction of Plasmids—For analysis of the intact TSHβ promoter, native and mutated sequences –106/+27-113 of the rat TSHβ gene were synthesized (Pharmacia Gene Assembler, Piscataway, NJ) with BamHI-HindIII linkers and subcloned into the polylinker of a promoterless CAT expression vector (PMV). Single point mutations were introduced at –8, –9, +18, +23, +27, intron +5, +13 (5 and 113, respectively) (Fig. 1). To analyze specific T3RE motifs, sequences were synthesized (Pharmacia Gene Assembler and Applied Biosystems 380B, Foster City, CA) with BamHI linkers and subcloned into the BamHI site (+106 relative to the TK transcriptional start site) of an expression vector PBLCAT8+ (Dr. W. Wahl, University of Lausanne, Lausanne, Switzerland). Uppercase letters indicate exonic sequences, whereas lowercase letters denote intron or upstream sequences. The TSH3 sequences included: –17/+27-113 (5′-agagtctgggtcatcacAGCA'M'AAC-3′); –17+/27-113 (5′-agagtctgggtcatcacAGCATTACCTGCAGAATGAC-3′); –9+/11 (5′-agagtctgggtcatcacAGCTAATGAC-3′); –11+/27-113 (5′-GGCCAGTCGAAGAATGAC-3′); +18+/27 (5′-GGCAGTAAG-3′); +113-/113 (5′-ggagtctgggtcatcacAGCATTACCTGCAGAATGAC-3′). Sequences not originating from rTSHβ included: TREPAL (5′-TCAAGAGTATACGTTAAGGAT-3′); TREPAL1/2 (5′-TCAAGAGTATACGTTAAGGAT-3′). All plasmid constructs were confirmed by DNA sequence analysis.

Cell Culture and Transfection—GH4 cells, kindly provided by Dr. Barry Brown, University of Sheffield, Sheffield, United Kingdom and COS cells (ATCC CRL 1651, American Type Culture Collection, Rockville, MD) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), glutamine (2 mM) (Life Technologies, Inc.). GH4 cells were transfected with TSHβ promoter plasmids (1–2 μg/× 106 cells/60-mm well) by (diethylaminoethyl-DEAE)-dextran (200 μg/ml) as described previously (11). For co-transfection studies, COS-7 cells were transfected as before (with T3R test plasmids (1–3 μg) in the absence or presence of PMT2 expression plasmids (0.5 μg)) containing coding regions of hT,Rα, hT,Rβ, RXRp (both T3Rα were gifts from Dr. Magnus Pihl, La Jolla Cancer Research Foundation, La Jolla, CA, and RXRp was given to us by Dr. K. Ozato, National Institutes of Health, Bethesda, MD). Transfections included equivalent amounts of plasmid DNA, PMT2 without cDNA sequence or in some cases with T3R in the antisense orientation were added as necessary.

Get Mobility Shift Assays—COS-7 cells were transfected with PMLT2 vectors containing cDNAs T3Rα and T3Rβ. Nuclear extracts were pre-
The inhibition, we introduced mutations within the T3RE half-site motifs are indicated by half-arrows. Selected mutations were introduced into each of three regions indicated. Basal expression is illustrated by the solid bars and activity in the presence of T3 (10^-8 M, 24 h) by the hatched bars. Data are summarized as mean ± S.E., n = 8 per group. Significant (p < 0.05) suppression by T3 compared with basal expression is indicated (*) and significant (p < 0.01) variation in basal expression is indicated (**).

**RESULTS**

**Location of Negative T3REs in rTSHβ Promoter DNA**—We have shown previously that two regions (-17/+9 and +11/+27) in the rTSHβ promoter mediate the negative actions of T3 on a heterologous promoter (6). To locate precisely the nucleotides responsible for the inhibition, we introduced mutations within these regions of the native TSHβ promoter (Fig. 1). The activity of the wild type promoter was reduced by 67% following 24-h exposure to T3. Mutations (Fig. 1A, thick horizontal arrows) that resembled the consensus T3RE half-site were targeted for the mutational analysis. Transverse mutations in -17/+9 region at positions -9 and -8 (Fig. 1, M-17/+9) reduced the negative effect of T3 to 50%. In contrast, mutations within the +11/+27 region (+18, +23, +27) eliminated sensitivity to T3 and slightly decreased basal expression (Fig. 1, M+11/+27). Mutations at intron sites 5 and 13 (Fig. 1, 11/13) failed to alter the inhibitory properties of the promoter in the presence of T3. Results arising from four separate transfections using each one of the constructs yielded data shown in the graph of Fig. 1B. Together these observations support the idea that a half-site motif can mediate the inhibitory effects of T3.

**Half-site Motifs of the T3REs Mediate Negative Regulation by T3**—The preceding results indicated that a limited region of the +11/+27 sequence mediated the negative actions of T3. To test this possibility single copies of both the +11/+27 sequence and a more limited region (+18/+27) were inserted in front of the viral TK promoter to yield TSHβ(+11/+27)TKCAT and TSHβ(+18/+27)TKCAT. In transfected pituitary GH3 cells, T3 decreased the promoter activity of both TSHβTKCAT chimeras (Fig. 2). As shown previously (6), TKCAT activity (percent total counts/min converted per mg of protein) was not different in the absence or presence (13.7 ± 2.3, 11.3 ± 3.1, respectively) of added T3 (10^-8 M, 24 h). This observation narrowed the inhibitory effects of T3 to the +18/+27 sequence of the rTSHβ nT3RE and raised the possibility that a half-site motif within this region mediated repressive actions of the hormone. To test the half-site hypothesis, we inserted the half-site motif of the idealized palindrome derived from the rGH T3RE (TREpal(U2)) in the 5'-flanking region of TKCAT to yield TREpal(1/2)TKCAT. As shown previously by numerous investigators, including ourselves, the intact TREpal conferred positive T3 regulation to a heterologous promoter (6). In GH3 cells transfected with TREpal(1/2)TKCAT, T3 decreased CAT activity in a dose-dependent manner (Fig. 2). Together these results support the idea that a half-site motif can mediate the inhibitory effects of T3.

**Presence of T3R Is Required to Mediate the Repressive Effects of T3**—The hormone-dependent inhibition of CAT activity in the transfected cells presumes that the negative effects of T3 were mediated by endogenous T3Rs present in GH3 cells. To verify that T3Rs were indeed involved in this process, we introduced the various reporter constructs into T3R-deficient COS cells. Although CAT activity in COS cells co-transfected with T3Ra and the parent vector, TKCAT, was unresponsive to T3, the expected increase was observed in cells co-transfected with the TREpalTKCAT construct (Fig. 3, A and B, respectively). T3 had no effect on TSHβ(+11/+27)TKCAT, TSHβ(+18/+27)TKCAT, or TREpal(1/2)TKCAT expression when transfected alone into the COS cells (Fig. 4). However, T3 suppression of CAT activity was restored when the templates were co-transfected with an expression vector (PMT2) containing the cDNA for either T3Ra or T3Rp. Co-transfection of either T3Ra or T3Rp enhanced basal expression of the TSHβTKCATs (Fig. 4, A and B) and TREpal(1/2)TKCAT (Fig. 4C) constructs without diminishing the negative response to T3. The ability of T3Ra or T3Rp to enhance basal expression has also been noted...
Inhibitory Effects of $T_3$

Fig. 2. Elements containing half-site motifs of the $T_3$RE mediate negative regulation by $T_3$ in transfected GH$_3$ cells. The rTSHp $T_3$RE +11/+27, +18/+27, or half-site of the idealized $T_3$RE palindrome (rGHTREPALV2) inserted as a single copy −108 to the TK transcriptional start site in the reporter vector PBLCAT8+ conferred a dose-dependent decrease in promoter activity in response to $T_3$. Data are mean ± S.E.; n = 6 per data point. Statistical significance at p < 0.05 is indicated relative to base line for each plasmid: +, * for rTSHp(+11/+27)TKCAT, rTSHp(+18/+27)TKCAT, and TREpal(1/2)TKCAT, respectively.

Fig. 3. Presence of a $T_3$R is required for the stimulatory effects of $T_3$. $T_3$R-deficient COS cells were co-transfected with 0.5 µg of P2 vector carrying cDNA for either $T_3$Ra or $T_3$Rb and/or RXRb and a reporter construct without (A) or with (B) a $T_3$RE. A reflects the activity of TKCAT (1 µg) in response to $T_3$ (10$^{-8}$ M, 24 h) in the absence (−) or presence (+) of the indicated plasmids. B shows the activity of TREpalTKCAT (1 µg) under the same treatments. Data are mean ± S.E.; n = 4 per group. Significance at p < 0.05 is indicated ** compared with basal control for each group.

on other TRE models (13). These results confirm that the inhibitory effects of the nT3RE required the presence of $T_3$R.

Recent studies indicating that a receptor for the 9-cis-retinoic acid (RXR) heterodimerizes with various $T_3$R isoforms prompted us to ask whether such an interaction might augment the function of nT3REs. We co-transfected RXRb with $T_3$Ra or $T_3$Rb and the $T_3$RE half-site templates into COS cells.

Fig. 4. Presence of a $T_3$R is required for the inhibitory effects of $T_3$. $T_3$R-deficient COS cells were co-transfected with 0.5 µg of P2 vector carrying cDNA for either $T_3$Ra or $T_3$Rb and/or RXRb and a reporter construct containing a nT3RE. A reflects the activity of rTSHp (+11/+27)TKCAT (1 µg) in response to $T_3$ (10$^{-8}$ M, 24 h) in the absence (−) or presence (+) of the indicated plasmids. B and C reflect the activity of rTSHp(+18/+27)TKCAT and TREpal(1/2)TKCAT, respectively. Data are mean ± S.E.; n = 8 per group. Significance at p < 0.05 is indicated ** compared with basal control for each group.
Inhibitory Effects of T₃

Unexpectedly, we found that co-transfection with RXRβ blunted or abolished the negative response to T₃ (Fig. 4). T₃R Isomers Bind to the T₃RE Half-Site Motifs—Finally, we examined whether both T₃R isomers bind to the TSHβ T₃RE. Nuclear extracts of transfected COS cells were tested for binding to the motif. Both T₃Rα and T₃Rβ bound to the radiolabeled nT3RE (Fig. 5). Competition with homologous DNA or TREpal(1/2) DNA abolished binding of the T₃R isomers to the radiolabeled probe, whereas addition of nonspecific DNA failed to compete. The major complex is a monomer (upper arrow) consistent with our previous studies on in vitro synthesized hT₃Rβ (6). Because we used COS cell extracts in this study, the possibility exists that the upper complex may be a heterodimer comprised of a single T₃R and a TRAP protein. A faster migrating complex (lower arrow) represents TSHβ-nT3RE binding activity present in untransfected COS cell extracts. Together these observations have shown that both T₃Rα and T₃Rβ bind to rTSHβ nT3RE and that homologous sequence or TREpal(1/2) serve as potent competitors.

Discussion

In this report, we present evidence that clearly shows a nonpalindromic half-site motif mediated the negative actions of T₃. Specific mutations to motifs resembling a consensus T₃RE half-site present in the context of the native rTSHβ promoter impaired or abolished the inhibitory effects of T₃. These findings extend our previous observations in vitro that mutations in these regions impair or completely block binding to GH3 nuclear protein extracts containing T₃Rs (6). That T₃RE half-site motifs were important features for negative regulation by T₃ was further demonstrated by T₃ suppression of TK promoter activity only in the presence of the rTSHβ DNA. Additional support for this hypothesis was obtained by examining the half-site from TREpal, a palindromic element that responds positively to T₃. In these studies, T₃ decreased TREpal(1/2)/TRECAT in GH3 cells.

The suppressive effects of T₃ required the presence of either the α or β isoforms of T₃R. Unexpectedly, we found that the additional presence of RXR blunts or abolishes T₃ inhibitory effects. One plausible explanation for this observation is that the known ability of RXRβ to heterodimerize with T₃Rα or T₃Rβ effectively sequesters T₃R in a form that restricts its interaction with the nT3RE. In contrast, RXRβ augments the stimulatory effects of T₃ and enhances binding to T₃REs that mediate positive effects of the hormone (14-16), as verified in results summarized in Fig. 3B.

How does this information fit into the current concepts of thyroid hormone action? The activity of the T₃RE in positively regulated genes or the canonical T₃RE appears to be dependent on the orientation, spacing of the half-sites, and context within the promoter (13, 17-19). The transcriptionally active form of the T₃R is considered to be a homodimer or heterodimer, as suggested by the presence of multiple half-site motifs and the loss of the T₃ stimulatory activity with mutation of one of the half-sites (20-22). The stimulatory activity of T₃ has been attributed to enhanced stability of heterodimer formation between T₃R and T₃R auxiliary proteins (TRAPs) and derepression of the dominant negative homodimers of T₃R (23-28). Although the preceding model may account for inductive effects of T₃ on gene activity, it does not explain the inhibitory effects of T₃ nor can it accommodate the findings in this report. These studies indicate that the half-site composition of the nT3RE does not conform to the homo- or heterodimer model of T₃ action. Both T₃R isoforms bind to the various T₃REs and mediate positive and negative regulation by T₃. Current data suggest that positive regulation by T₃ is mediated by a palindrome such as TREpal or imperfect repeats as present in rGH DNA. In contrast, negative regulation appears to be mediated by half-site motifs as reflected in TSHβ and other genes inhibited by T₃ (6-10, 13, 29-33). T₃Rs bind as either homo- or heterodimers to positive T₃REs, but only a monomer or heterodimer can bind to the half-site nT3RE (18, 17-19, 23-28).

Based on these observations, we speculate that for nT3REs, in the absence of T₃, T₃R has a neutral or a positive effect on transcription. Addition of T₃ alters the conformation of the T₃R bound to DNA such that it cannot activate transcription. Another possibility is that exposure to T₃ may stabilize the T₃R-TRAP interaction such that it is sequestered from interactions with the nT3RE. Such a model is supported by our observation that RXRβ abolishes the inhibitory effects of T₃. Both models may be functional depending upon the specific sequence and context of individual T₃REs.

In summary, positive regulation by T₃ is mediated by an idealized T₃REpal or imperfect repeats, whereas negative effects reside in a half-site motif found in TSHβ and other genes suppressed by T₃ (7-10). The binding of T₃R homo- or heterodimer to the positive elements and a monomer to nT3RE may account for the differential actions of T₃. Based on these observations, we propose that monomer T₃R binding to a half-site motif in the absence of T₃ exerts a neutral or a positive effect on transcription. Addition of T₃ alters the conformation of the T₃R bound to DNA, leading to a change in activity possibly by facilitating interaction with TRAP proteins (activator or derepressor) or by destabilizing the T₃R-DNA complex and in turn disrupting the transcription initiation complex. These models are not mutually exclusive and may contribute to the overall process of negative regulation by T₃, depending on the specific nT3RE and the surrounding elements in the context of the native gene.

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