Negative and Positive Transcriptional Regulation by Thyroid Hormone Receptor Isoforms

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Multiple forms of human thyroid hormone (T_3) receptor have been identified, including true receptors that bind T_3 (α_1 and β) and a splicing variant (α_2) that does not bind T_3. The α_1- and β-receptors activate transcription through interactions with positive thyroid response elements (TREs). The α_2 variant is unable to activate transcription and has been reported to inhibit α_1 or β stimulation of positive TREs, a property referred to as dominant negative activity. In this report we have performed studies to assess the functional properties of different members of the thyroid receptor family with regard to both positive and negative transcriptional regulation. The α_1-, α_2-, and β-receptors were each coexpressed in JEG-3 cells with either TreTKCAT (CAT = chloramphenicol acetyltransferase), a reporter gene that contains a positive TRE, or TSHαCAT, a negatively regulated reporter gene. The α_1 and β isoforms stimulated transcription of TreTKCAT and inhibited TSHαCAT transcription in a T_3-dependent manner, whereas the α_2 variant was inactive. When coexpressed with α_1- or β-receptors, α_2 inhibited regulation of positive TREs, but the effects of α_2 were modest and only occurred when relatively high doses of receptor were transfected. The α_2-receptor variant did not affect negative regulation by α_1- or β-receptors. Thus, in both positive and negative regulation, thyroid hormone receptor isoforms that bind T_3 (α_1,β) are functional, whereas the α_2 isoform, which does not bind T_3, is not functional. The inhibitory properties of α_2 are dependent on total receptor dose for positive regulation and are not evident in negative regulation. These data are consistent with a mechanism of inhibition in which α_2 interacts with a limiting nuclear factor(s) that is required for receptor function or transcriptional stimulation. (Molecular Endocrinology 4: 1522–1531, 1990)

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INTRODUCTION

Thyroid hormone (T_3; 3,5,3′-L-triiodothyronine) regulates development and metabolism, acting via a family of thyroid hormone receptors to modulate the transcription of thyroid hormone-responsive genes. In current models for thyroid hormone action, T_3 binds to its intranuclear receptors to either stimulate or repress transcriptional activity by interactions with specific thyroid response elements (TREs) of target genes (1, 2).

Thyroid hormone receptors are members of a superfamily of receptors that also includes the steroid hormones, vitamin D, and retinoic acid receptors (1). Each member of the superfamily has a central conserved DNA-binding domain and a separate carboxy-terminal ligand-binding domain (1). Additional modular domains may exist to convey properties of nuclear localization, receptor dimerization, and transcriptional activation (3), but these areas have not been clearly delineated in T_3 receptors.

Two major subtypes of T_3 receptors, designated α (chromosome 17) (4) and β (chromosome 3) (5), have been described. Within the α group there are at least two distinct transcripts, α_1 (4, 6) and α_2 (7–9). The first 370 amino acids of α_1 and α_2 are identical, but the carboxy-terminal 40 residues of α_1 and 120 residues of α_2 are distinct. The site of divergence between α_1 and α_2 includes a consensus donor sequence for an exon-intron junction, and it has been proposed that α_1 and α_2 are splicing variants (8, 10). Unlike the α_1- and β-receptor isoforms, α_2 does not bind T_3 (11). Thus, α_2 is not formally a T_3 receptor. However, since it contains a large segment of receptor sequence, α_2 will be referred to subsequently as a thyroid hormone receptor isoform.

In the context of positive transcriptional regulation by thyroid hormone receptors, it has been shown that the α_1- and β-receptors are each capable of transcriptional stimulation in the presence of T_3 (10, 12, 13), whereas the α_2 isoform is inactive (14). However, the α_2 isoform

has been shown to inhibit positive transcriptional responses mediated by the α_1- and β-receptors (14, 15). These findings have led to the suggestion that the α_2 isoform may act to impair positive regulation by thyroid hormone in a dominant negative manner.

We have recently described a transient expression system for examining negative transcriptional regulation of the α-subunit gene of TSH by thyroid hormone. Deletion analyses define a negative TRE within the first 100 basepairs (bp) of the TSHα promoter, and a receptor-binding site was identified between −22 and −7 bp relative to the transcriptional start site (16).

In this report we have examined the function of the α_1-, α_2-, and β-receptor isoforms with respect to both positive and negative regulation by thyroid hormone. Furthermore, we have examined the ability of the α_2 isoform to interact with the functional T_3 receptors to alter their regulation of T_3-responsive genes.

RESULTS

Expression and Binding Properties of α_1-, α_2-, and β-Receptor Isoforms

The sizes, relevant domains, and T_3-binding properties of the receptor isoforms are depicted in Fig. 1A. α_1, α_2, and β all contain structurally similar DNA-binding domains and have been shown to bind to TREs *in vitro* (10, 17, 18). There is general agreement that only α_1- and β-receptors bind T_3 with high affinity, whereas the binding properties of α_2 have been controversial (7–9, 11, 18). Because the thyroid hormone-binding properties of the T_3 receptor isoforms have been disputed, we examined the abilities of the human α_1, α_2, and β isoforms to bind T_3 *in vitro* and *in vivo*.

To measure T_3 binding to receptor protein, α_1, α_2, and β were transcribed and translated *in vitro*. The proteins generated were incubated with 0.1 nM ^{125}I-labeled T_3 in the absence or presence of a 10,000-fold excess of unlabeled hormone (Fig. 2). Whereas α_1 and β exhibited comparable high affinity and saturable T_3 binding, α_2 binding was indistinguishable from background. These data confirm previously described T_3-binding properties of human α_1- and β-receptors (4, 5, 11) and support the view that the human α_2 variant does not bind T_3 (8, 11, 18).

Extracts of JEG-3 cells transfected with CDM α_1, α_2, or β were analyzed for binding to labeled DNA using gel mobility shift assays. Although the transfected receptors were functional, as assessed by parallel transient expression assays (see below), the level of receptor binding to DNA was below detection (data not shown), presumably due to the relatively small fraction of JEG-3 cells that are transfected. The CDM α_1, α_2, and β expression vectors were, therefore, transfected into another thyroid hormone receptor-deficient cell line (COS-1) to demonstrate that each of the receptors was expressed and to assess the DNA-binding properties of each receptor form. COS-1 cells express the simian

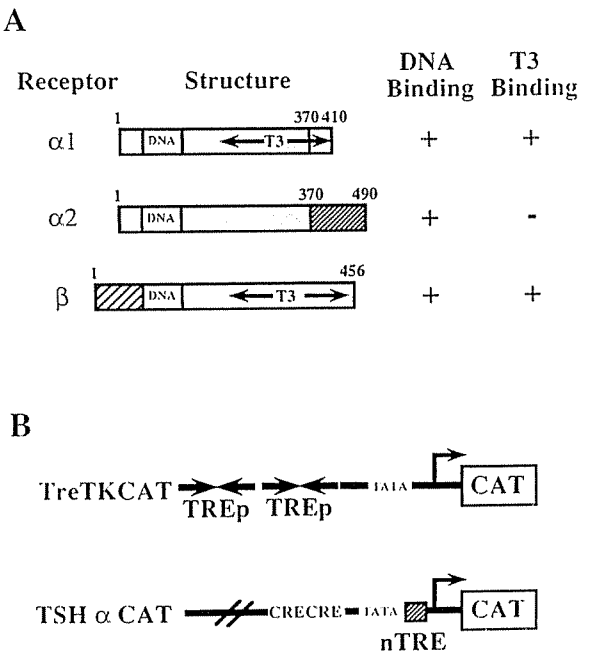

Fig. 1. Structures of T_3 Receptor Isoforms and Reporter Genes

A, Schematic representation of human α_1, α_2, and β T_3 receptor isoforms. Putative DNA (□)- and T_3 (*arrows*)-binding domains are indicated. The α_1 and α_2 isoforms are identical between amino acids 1 and 370, but diverge completely in the carboxy-terminal region. Although the α_1 and β isoforms differ at the amino-terminus, the receptors are highly homologous throughout the remainder of their sequence. The DNA-binding and T_3-binding properties of the receptors are indicated at the *right* of the figure. B, Reporter gene constructs used in transient expression assays. In TreTKCAT, the location of two copies of a positive TRE (TREp) is indicated by *arrows*. In TSHαCAT, the positions of two cAMP response elements (CRE) and the putative negative TRE (nTRE; ▨) are diagrammed. The locations of the TATA box, transcriptional start site, and the CAT gene are also indicated for both reporter constructs.

virus-40 T-antigen, causing high copy amplification of transfected CDM8 plasmids and allowing high levels of receptors to be expressed (19). Whole cell extracts of transfected COS-1 cells were incubated with ^{32}P-labeled palindromic TRE (TREp) that contains a known receptor-binding site (17). Receptor-DNA complexes were separated by electrophoresis on a nondenaturing gel (Fig. 3A). In cells transfected with the parent CDM8 plasmid, there was minimal protein binding to TREp. After transfection with the α_1-containing vector, extracts generated two new prominent bands with TREp. The compositions of the major and minor bands in the α_1 extracts are not known at present. Transfection with the β-receptor also resulted in two specific receptor-DNA complexes, which characteristically migrated with slower mobility than α_1 complexes.

The α_2 isoform also formed two complexes with TREp, but its binding properties were different from those of the α_1- and β-receptors. In contrast to α_1 and β, a substantial amount of the DNA-α_2 protein complex

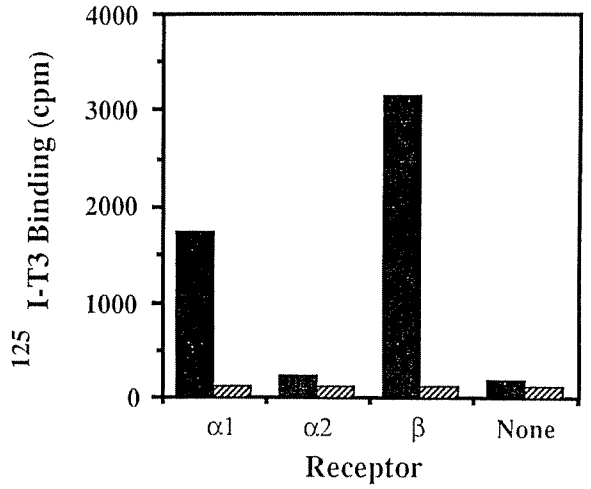

Fig. 2. T_3-Binding Properties of α_1-, α_2-, and β-Receptor Isoforms

Human α_1-, α_2-, and β-receptor cDNAs were transcribed and translated *in vitro* and assayed for binding to [^{125}I]T_3 (0.1 nM) in the absence (■) or presence (▨) of excess (10,000-fold) unlabeled T_3.

failed to enter the gel, and the α_2 complexes appear to dissociate during electrophoresis. These properties of α_2 have been reproduced in three separate extracts. Thus, all three isoforms are expressed by the CDM8 vector, but α_2 interactions with DNA are qualitatively different from α_1- and β-receptors.

The binding of T_3 was assessed for each receptor form by incubating the COS-1 cell extracts with ^{125}I-labeled T_3 and performing gel mobility shift assays with unlabeled TREp or with a control oligonucleotide. For both α_1 and β, [^{125}I]T_3-labeled receptor-DNA complexes comigrated with ^{32}P-labeled TREp complexes, confirming that these complexes contain T_3 receptors (Fig. 3B). Specific complexes were not apparent in control lanes that contained extracts from untransfected cells or in the reactions that contained receptor and control DNA. In contrast to extracts transfected with α_1- and β-receptors, α_2-containing complexes were not detected by labeling with [^{125}I]T_3, consistent with absence of T_3 binding to this isoform.

Transcriptional Regulation of Reporter Genes by α_1, α_2-, and β-Receptor Isoforms

Two reporter genes (Fig. 1B) were used to assess the properties of the receptor isoforms with respect to positive and negative regulation. TreTKCAT (CAT = chloramphenicol acetyltransferase; TK = thymidine kinase) contains two copies of an optimized positive TRE placed upstream of the TK reporter and CAT gene. TSHαCAT contains the promoter region of the TSHα gene, which contains a negative response element placed upstream of the CAT gene. CDM8 expression vectors containing the cDNAs encoding human α_1, α_2, and β were each transfected with a reporter construct into the thyroid hormone receptor-deficient JEG-3 choriocarcinoma cell line. After incubation in the presence or absence of T_3, induction or repression of transcriptional activity by each receptor isoform was assessed by measuring CAT activity.

The α_1, α_2, and β isoforms were first tested for their ability to induce positive regulation in this cotransfection system. Native JEG-3 cells or those transfected with the CDM8 vector alone minimally stimulated TreTKCAT (2.1-fold) in the presence of 5 nM T_3 (Fig. 4A), which is consistent with previous studies showing that this cell line is deficient in functional thyroid receptors (14, 16). After cotransfection of α_1 or β, basal transcriptional activity (in the absence of T_3) of TreTKCAT was diminished compared with that of a control plasmid that does not express receptor (CDM8). After treatment with T_3, the α_1- and β-receptors caused approximately 100-fold stimulation relative to basally repressed CAT activity, and 70- to 80-fold stimulation relative to control CDM8 transfections. The α_2 splicing variant exhibited less basal repression than α_1- or β-receptors and elicited no more T_3 responsiveness than the control plasmid.

Similar analyses of the functional properties of the receptors were performed with regard to negative regulation. In the absence of transfected receptors, TSHαCAT consistently exhibited some degree of T_3-mediated repression (20–50% suppression in different experiments; Fig. 4B). T_3-mediated suppression in the absence of cotransfected receptors may reflect the presence of a low amount of endogenous receptors. In contrast to the responses with TreTKCAT, transfected receptors had no effect on basal TSHαCAT expression in the absence of T_3 (Fig. 4B). Thus, basal repression or activation by receptor without T_3 is not evident for the negative TRE. After treatment with T_3, expression of α_1- or β-receptors caused 90% inhibition of TSHαCAT transcription (Fig. 4B). Relative to the vector alone, expression of α_2 did not confer T_3-mediated suppression of TSHαCAT. Thus, the α_1 and β forms of thyroid hormone receptor are capable of T_3-induced transactivation of positive TREs and repression of negative TREs, but the α_2 splicing variant does not mediate positive or negative transcription in response to T_3.

Dose Response for α_1-, α_2-, and β-Receptor Effects in Transfected JEG-3 Cells

Dose-response analyses were performed to define the parameters for experiments in which various combinations of receptors were expressed (Fig. 5). JEG-3 cells were cotransfected with reporter plasmid and increasing amounts of each receptor isoform in the presence or absence of 5 nM T_3. Using TreTKCAT, the reporter for positive transcriptional activation, 50% maximal activation was observed using 20 ng α_1 and 35 ng β, respectively. For both receptors, T_3 responsiveness reached a plateau after transfection of 100 ng receptor, suggesting that at a certain level of receptor, the degree of responsiveness may be limited by other factors. The α_2 isoform, at any dose, was no more active than the CDM8 plasmid alone. Thus, relatively small amounts of

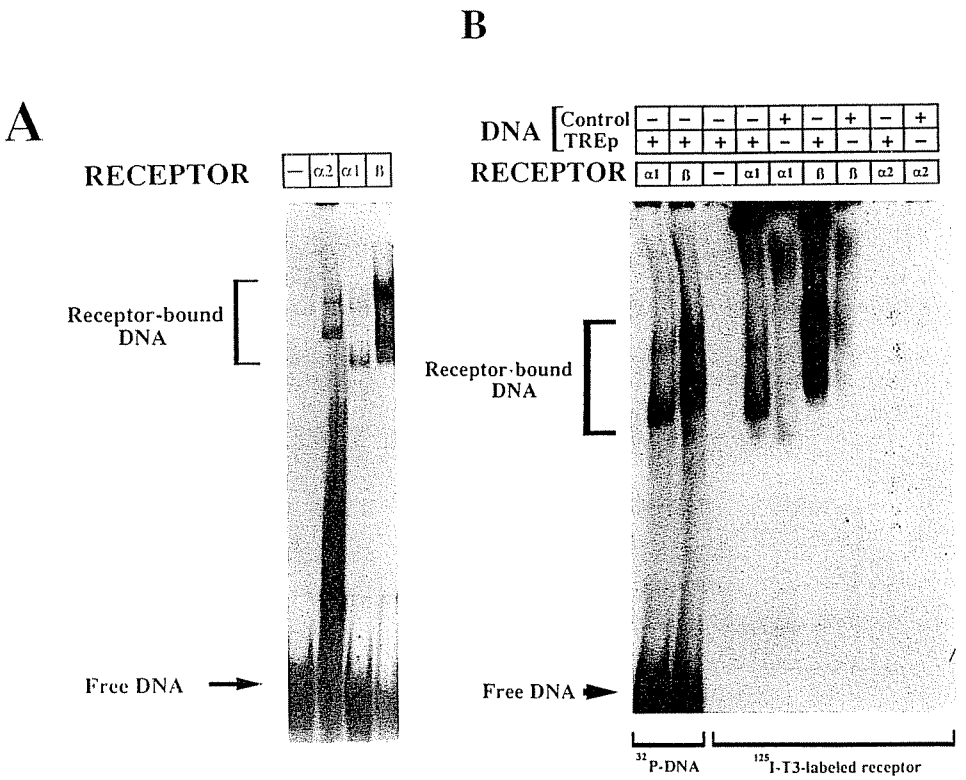

Fig. 3. Expression of CDMα_1-, α_2-, and β-Receptors in COS-1 Cells

Receptor expression vectors were transiently expressed in COS-1 cells, and cellular extracts were prepared as described in *Materials and Methods*. A, Receptor binding to ^{32}P-labeled DNA using gel mobility shift assays. COS-1 extracts containing the indicated receptors were incubated with ^{32}P-labeled TREp, and receptor-DNA complexes were analyzed by gel mobility shift assays. Free (*arrows*) and receptor-bound (*bracketed*) DNA fragments are indicated. B, T_3 binding to receptors expressed in COS-1 cells. COS-1 extracts containing the indicated receptors were incubated with ^{125}I-labeled T_3. After binding to either unlabeled TREp or a control DNA fragment (−132 to −92 bp) from the TSH α promoter, receptor-DNA complexes (*bracketed*) were analyzed by gel mobility shift assays. For comparison, receptor-DNA complexes formed using ^{32}P-labeled DNA are shown at the *left* of the figure.

expression vector containing functional thyroid hormone receptor are required to achieve maximal activation of TreTKCAT, and the α_2 splicing variant is unresponsive to T_3.

Analogous dose-response experiments were performed using the negative TRE-containing reporter, TSHαCAT. Half-maximal repression occurred using 20 ng α_1 and 30 ng β, with maximal repression occurring with 100 ng receptor. Using α_2 at varying doses, repression was no greater than with CDM8 alone, confirming the inability of α_2 to effectuate negative regulation.

Effects of α_2 on α_1- and β-Mediated Transcriptional Regulation

Previous studies have shown that the α_2 isoform inhibits positive transcriptional responses mediated by the α_1- and β-receptors (14, 15). To further evaluate this property of α_2, we performed parallel experiments using TreTKCAT and TSHαCAT as reporter genes for positive and negative regulation, respectively. Based on the results of the dose-response experiments, 200 ng receptor expression vector (α_1 or β) was selected as an amount sufficient to elicit full *trans*-activation of TreTKCAT. For competition, 1 μg α_2, 5-fold the amount of receptor vector, was cotransfected with 200 ng α_1 or β-receptor. Under these conditions, α_2 had little, if any, effect on the regulation of TreTKCAT by α_1- or β-receptors. For example, comparable T_3 activation of TreTKCAT was found after transfection of the α_1- or β-receptors alone (65- to 80-fold induction) or in the presence of a 5-fold excess of α_2 (55- to 70-fold induction; Fig. 6; Low Dose).

The absence of α_2 isoform inhibition of positive regulation was unexpected. The effects of α_2 were, therefore, examined using the same ratios of α_2/β and α_2/α_1, but at higher total receptor doses to more closely approximate the conditions used in other studies (14, 15) (Fig. 6, High Dose). Using 1 μg α_1- or β-receptor expression vector, 80- to 100-fold stimulation was observed, which was a greater response than the 60-fold stimulation observed at lower receptor doses. The increase in the fold stimulation at the higher dose was due to a larger degree of basal repression by receptor in the absence of T_3. When a 5-fold excess (5 μg) of α_2 was cotransfected with α_1 or β, the level of T_3-stimu-

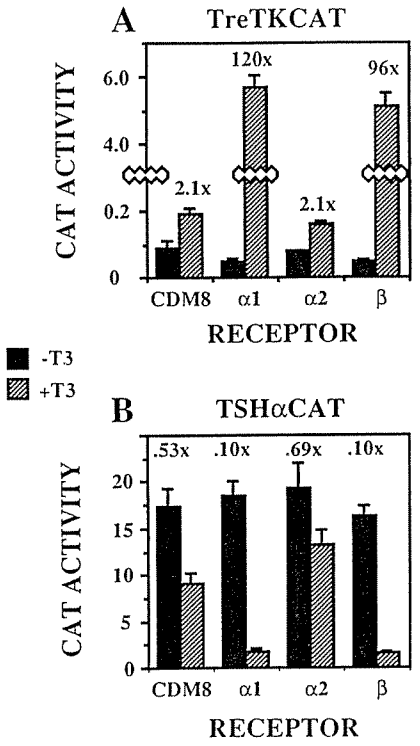

Fig. 4. Functional Properties of the T_3 Receptor Isoforms

Control plasmid (CDM8) or each of the indicated receptor isoforms cloned into CDM8 (1 μg) were transfected into JEG-3 cells with 5 μg of the indicated reporter gene (TreTKCAT or TSHαCAT). Triplicate sets of transfections were incubated in either the absence (■) or presence (▨) of 5 nM T_3. CAT activity is expressed as the percent chloramphenicol acetylation per μl cell extract/h. Relative stimulation or repression in the presence of T_3 treatment is indicated *above the bars*. The data are the mean ± SD. Similar results were obtained in five separate experiments. Note that the scale of the *ordinate* in A is broken to allow adequate visualization of receptor effects on CAT activity in the absence of T_3. A, Positive regulation of TreTKCAT by T_3. B, Negative regulation of TSHαCAT by T_3.

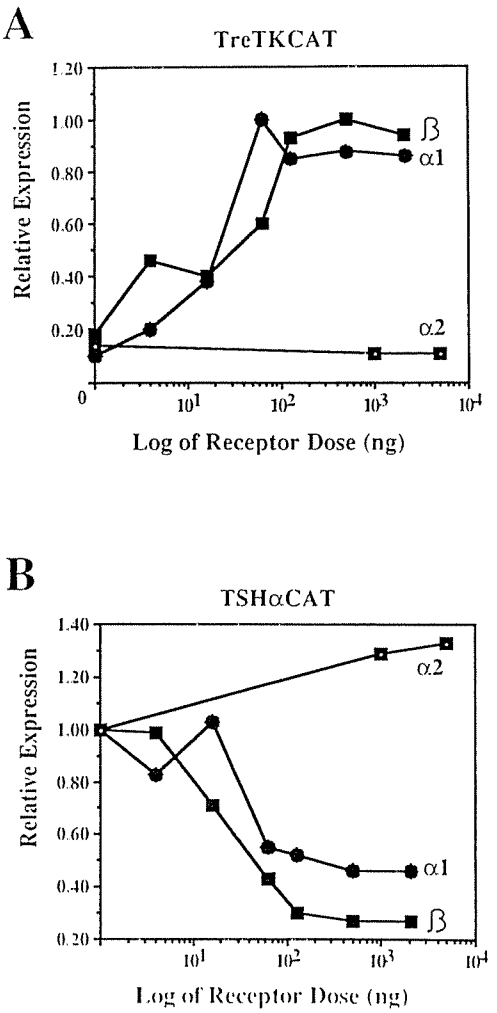

Fig. 5. Dose Response for Transcriptional Regulation by Transfected Receptors

Increasing amounts of CDMα_1, α_2, and β were transfected into JEG-3 cells with the indicated reporter constructs (TreTKCAT or TSHαCAT; 5 μg). The expression vector CDM8 was added to each transfection to maintain a constant total amount of plasmid. *Data points* are the mean of duplicate transfections. Similar dose-responses were observed in three independent experiments. A, Positive regulation of TreTKCAT by T_3. B, Negative regulation of TSHαCAT by T_3.

lated activity was reduced by 30–50%. The inhibition by α_2 was due primarily to a decrease in the T_3-stimulated component of activation, as the addition of high doses of α_2 appears to act additively to further repress basal transcription.

The same experimental paradigm was employed for analyses of the negative reporter, TSHαCAT (Fig. 7). Under low dose conditions (200 ng receptor DNA), 80–90% suppression was observed after treatment with T_3 (Fig. 7, Low Dose). The same level of inhibition was seen when excess α_2 was cotransfected with either the α_1- or β-receptors.

Because α_2 affected positive regulation only at higher total doses of receptor, negative regulation of TSHαCAT was also examined under these conditions (Fig. 7, High Dose). However, even at higher doses of total receptor (*e.g.* β, 1 μg; α_2, 5 μg), α_2 did not alter the degree of T_3-mediated repression by α_1- or β-receptors. Thus, α_2 does not affect TSHαCAT repression by cotransfected functional receptors, regardless of the total amount of transfected receptor.

DISCUSSION

In this report we describe the properties of different thyroid hormone receptor isoforms in their control of positive and negative transcriptional regulation. The results suggest three conclusions about human thyroid hormone receptor function. First, the α_1 and β isoforms encode thyroid hormone receptors that are each capable of mediating both positive and negative transcrip-

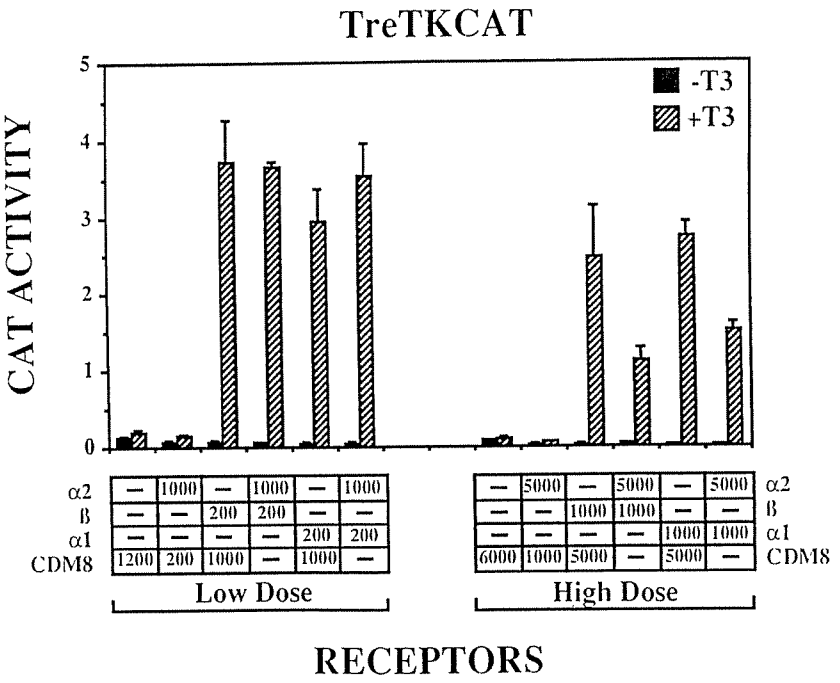

Fig. 6. Effects of the α_2-Receptor Isoform on Positive Regulation of TreTKCAT by α_1- and β-Receptors

TreTKCAT (5 μg) was cotransfected into JEG-3 cells with the indicated combinations of receptor expression vectors with either low doses (1200 ng) or high doses (6000 ng) of total receptor. Triplicate sets of transfections were incubated in the absence (■) or presence (▨) of 5 nM T_3. The data are the mean ± SD. Similar results were obtained in three independent experiments.

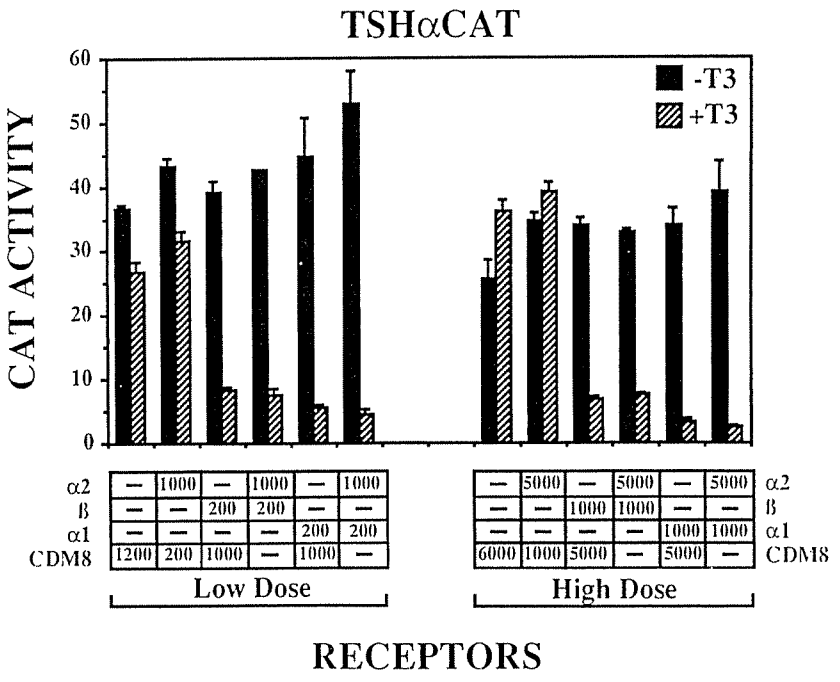

Fig. 7. Effects of the α_2-Receptor Isoform on Negative Regulation of TSHαCAT by α_1- and β-Receptors

TSHαCAT (5 μg) was cotransfected into JEG-3 cells with the indicated combinations of receptor expression vectors with either low doses (1200 ng) or high doses (6000 ng) of total receptor. Triplicate sets of transfections were incubated in the absence (■) or presence (▨) of 5 nM T_3. The data are the mean ± SD. Similar results were obtained in three independent experiments.

tional regulation in a thyroid hormone-dependent manner. Second, α_2 encodes a structural variant that is unable to bind thyroid hormone, and it does not elicit either positive or negative regulation of TRE-containing reporter genes. Third, coexpression of the α_2 isoform with the α_1- or β-receptors reveals that α_2 has inhibitory activity for positive regulation, but only when expressed at relatively high total doses of receptor. The α_2 isoform has no effect on receptor suppression of a negatively regulated gene.

Initially, the α_2 isoform was reported to bind T_3 (7, 9). Subsequently, several groups reported that T_3 binding to the α_2 splicing variant was markedly reduced or nonexistent (8, 11, 15, 18). Our *in vitro* binding studies confirm that α_2 does not bind T_3 (Fig. 2). Furthermore, after expression in COS-1 cells, the α_1- and β-receptors were shown to bind T_3, whereas the α_2 isoform did not (Fig. 3). Thus, the absence of T_3 binding is not the result of using the *in vitro* transcription/translation assay system, and it seems likely that the carboxy-terminal sequence variation in α_2 either eliminates critical residues that are required for T_3 binding or creates a peptide domain that restricts T_3 binding.

Because we were unable to demonstrate transcriptional regulation by the α_2 isoform in transient assays, it was important to demonstrate that the CDMα2 expression vector was functional. Assays of receptor binding to a high affinity TRE-binding element were used as an indirect measure of receptor expression. As shown in Fig. 3, the proteins expressed from CDM8 vectors containing α_1 and β specifically bound a TREp, creating at least two distinct complexes. The complexes may represent monomer and dimer forms of T_3 receptors or, alternatively, T_3 receptor interactions with other cellular factors. Extracts from cells transfected with α_2 also bound to TREp, confirming that α_2 is expressed by this vector. Similar data were observed using a negative TRE (nTRE) from the TSHα promoter (16), except that the binding interactions were of lower affinity, requiring 50-fold greater concentrations of DNA for comparable receptor binding (Datta, S., unpublished data). Although α_1 and α_2 share an identical DNA-binding domain, α_2 interactions with TREp were distinctly different from those of α_1 or β. First, in several independent preparations, α_2-containing extracts formed aggregates at the top of the gel, suggesting that α_2 either self-associates or interacts with other cellular components in a manner that is distinct from the T_3 receptors (Fig. 3 and data not shown). Second, the α_2-TREp complexes appear to dissociate during electrophoresis, causing a smeared appearance, potentially reflecting reduced affinity of α_2 for the TRE. The issues of receptor affinities for DNA and potential interactions with other cellular factors will require additional studies using purified receptor isoforms.

As other investigators have described (20, 21), we found that in the absence of T_3, the α_1- and β-receptors caused repression of the positive TRE-containing vector TreTKCAT. These data suggest that the receptors may interact with DNA in the absence of T_3 to exert an inhibitory effect on transcription that is opposite from the transcriptional activation that occurs after binding of T_3. Since α_2 contains an intact DNA-binding domain that is capable of interactions with a TRE, it seemed likely that α_2, like α_1 and β, might cause basal repression even though it does not bind T_3. By analogy, v-*erb*-A, a viral homolog of the thyroid receptor that lacks a complete carboxy-terminus and cannot bind T_3, causes basal repression of a positive TRE (22). Although α_2 consistently caused a small degree of basal repression, it was less than that observed with α_1- or β-receptors (Fig. 4). One cannot determine from these experiments whether α_2 binds DNA with lower affinity, or whether its ability to mediate basal repression is impaired by the carboxy-terminal extension. It is also possible that α_2 interacts with other cellular components to restrict its access to DNA. There may be a functional parallel between the altered interactions of α_2 with the TREp *in vitro* and the reduced basal repression by α_2 in transfected cells.

The observation that T_3 receptors repress a positively regulated TRE in the absence of thyroid hormone raises the issue of how the receptors might affect a negatively regulated gene in the absence of hormonal signals. For example, will the receptors act as constitutively activated transcriptional repressors by virtue of binding to a nTRE? Or, in analogy with positive regulation, will the receptors act in an opposite manner in the absence hormone, causing basal activation in the absence of T_3 and repression in the presence of T_3? For TSHαCAT, no basal effects of receptors were observed, and transcriptional repression was seen only after T_3 binding to receptor. Furthermore, the α_2 isoform did not function as a constitutively active repressor or as an activator of negative regulation (Fig. 4). It is possible that the absence of basal repression of TSHαCAT by T_3 receptors or α_2 reflects a relatively lower affinity of the nTRE for receptor isoforms.

The biological function of the different T_3 receptor isoforms is unclear at the present time. One possibility is that the α_1 and β isoforms might differentially regulate positive or negative transcription. We have previously found that the β-receptor can mediate both positive and negative regulation in a T_3-dependent manner (16). We now find that the α_1-receptor is also capable of both positive and negative regulation (Fig. 4). Furthermore, the degree of T_3-mediated stimulation (TreTKCAT) or repression (TSHαCAT) was comparable for both forms of receptors (Fig. 4). In additional experiments, we found that cotransfection of the α_1- and β-receptors resulted in additive effects on transcriptional stimulation or repression. Thus, at least when assessed by transient expression assays, the α_1- and β-receptors appear to be interchangeable. In view of the similar functional properties of the receptors, the role of tissue-specific expression of the α- and β-genes provides an intriguing mechanism for expression of receptors in response to different physiological signals. Clearly, it also remains possible for the α_1- and β-receptors to selectively interact with other cellular factors or target

genes, or to elicit subtle forms of transcriptional regulation that are not apparent in transient assays.

Cotransfection of α_2 with either α_1- or β-receptors has been reported to inhibit T_3-mediated stimulation of a positively regulated reporter gene (14, 16). Similarly, the oncogene v-*erb*-A, an inactive thyroid hormone receptor variant, also acts in dominant negative manner when transfected with T_3 receptor DNA (21, 22). Thus, the α_2 splicing variant may function as an endogenous inhibitor of thyroid hormone receptor action. In view of these studies, we examined whether α_2 also inhibited receptor-mediated transcriptional repression. We found that α_2 had little or no effect on α_1- or β-receptor repression of TSHαCAT (Fig. 7). This observation led us to reevaluate the effects of α_2 on receptor stimulation of positively regulated gene under our experimental conditions. We were unable to demonstrate α_2 inhibition under the conditions of our transfections, which used maximally active doses of transfected receptors (200 ng) and a 5-fold excess of α_2. Because previous studies used higher doses of transfected receptors, the effects of total receptor dose on α_2 inhibition were examined while maintaining a constant ratio of α_2/α_1 and α_2/β. These experiments revealed that α_2 inhibited positive regulation, but only under high dose receptor conditions. The effect of α_2 under these conditions is primarily on the absolute level of T_3-stimulated expression. Because transfection of excess α_2 increased the degree of receptor-mediated basal repression, α_2 had a smaller effect on fold stimulation after T_3 treatment. The α_2 isoform had no reproducible effect on receptor-mediated repression of a negatively regulated gene, under either low or high dose conditions (Fig. 7). Thus, the inhibitory effects of high dose α_2 are specific for the positively regulated reporter gene.

At least three possible mechanisms might account for transcriptional inhibition by α_2. First, α_2 could compete for receptor binding to DNA. In this model, α_2 would bind to the TRE and sterically hinder binding of functional receptors. In support of this type of mechanism, it has been shown that DNA-binding mutations in v-*erb*-A preclude its function as aa dominant negative inhibitor (21). In addition, the *in vitro* binding studies suggest that α_2 may interact with DNA less effectively than α_1- or β-receptors. Although this mechanism cannot be excluded, one might predict that a 5-fold excess of transfected α_2 would have caused equally effective inhibition under conditions of both low and high doses of total receptor. However, if the concentration of α_2 must exceed a threshold level before it causes inhibition, competition for receptor binding to DNA would be compatible with the observed results.

A second mechanism for α_2 inhibition would be for it to form an inactive heterodimer with functional α_1- or β-receptors. Although there is little direct evidence for T_3 receptor homodimers or heterodimers, some TREs have dyad symmetry, and T_3 receptors have been shown to form heterodimers with retinoic acid receptors (23). In support of this model, mutant T_3 receptors that only contain the carboxy-terminal domain have been shown to inhibit the function of wild-type receptors (24). Because these mutants lack the DNA-binding domain, they have been proposed to impair receptor function by forming heterodimers via interactions of α-helical heptad repeats of hydrophobic residues in the carboxy-terminal receptor domain. This mechanism for dominant negative activity would also predict that inhibition by excess expression of α_2 would be equally effective regardless of the total dose of receptor. Moreover, one might expect that the dominant negative effects of inactive heterodimers would be observed with respect to both positive and negative transcriptional regulation. In support of this concept, we have recently found that a variety of β-receptor mutants that preclude T_3 binding can function as potent inhibitors of both transcriptional activation and repression, and that the inhibitory effects are fully manifest under the low dose transfection conditions (Chatterjee, V. K. K., unpublished data).

A third mechanism for α_2 inhibition would involve α_2 interactions with a limiting factor that is required for either receptor function or transcriptional activation. A number of recent studies have demonstrated a critical role for adaptor molecules in transcriptional regulation (25). These factors, which interact with both enhancer binding proteins and basal transcription factors such as TFIID, can be readily depleted in some instances via binding to other transcription factors. In several respects our data are most compatible with this mechanism. Firstly, the dose-response curves for transfected receptors indicate that a plateau in transcriptional activation was attained after transfection of about 100 ng expression vector. The basis for this plateau in transcriptional activity is unknown, but a limiting amount of critical accessory factors could be responsible for the restriction of receptor-mediated expression. The presence of a limiting accessory factor could also account for the dose-related effects of α_2 that were observed in the competition experiments. At low receptor doses, there may be a sufficient amount of the accessory factor available for the active receptor to function. At higher receptor doses, excess α_2 may bind enough of the factor to arrest transcriptional activation. The α_2 isoform might not impair negative regulation because different mechanisms and different factors are probably involved, so that only a factor involved in transcriptional activation would be titrated out by α_2. The properties of α_2 observed in the gel mobility shift assays may reflect its interactions with one or more accessory factors (Fig. 3A), some of which may include the proteins that have been shown to enhance the binding of thyroid hormone receptors to TREs (26, 27).

The physiological significance of multiple thyroid hormone receptor isoforms remains unknown, and in particular, the biological function of the non-T_3-binding α_2-receptor isoform is intriguing. It is possible that the α_2 isoform interacts with a ligand that has not yet been identified and that this ligand selectively activates the α_2 isoform. Although our experiments suggest that α_2 is not a potent dominant negative inhibitor of receptor function in transient expression experiments, there is

evidence to suggest that this splicing variant interacts with other cellular factors that are required for maximal transcriptional activation of a positive TRE. The functional properties of α_2 may differ from those of other reporter genes, and it is also important to note that the relative amounts of α_1, α_2, and β isoforms vary widely in different cell types (28), a feature that may provide a mechanism for differential tissue responses to thyroid hormone.

MATERIALS AND METHODS

Plasmid Constructions

TSHαCAT contains 846 bp of 5′-flanking sequence and 44 bp of exon I of the human glycoprotein hormone α gene linked to the gene encoding CAT (29). TreTKCAT (provided by G. Brent and D. Moore, Massachusetts General Hospital, Boston, MA) contains two copies of a palindromic TRE (5′-gatcTCAGGTCATGACCTGAgatc-3′; linker sequences are shown in small case letters) inserted up-stream of the herpes simplex virus TK promoter linked to the CAT gene (13). *Hin*dIII linkers were used to insert the human *erb*Aα_1 (30) and -β (6) cDNAs into pCDM8, a vector in which expression is driven by the cytomegalovirus promoter (19). CDM8α_2 was created by transferring an *Xba*I fragment that contains the α_2-specific carboxy-terminal sequence (9) into CDM8α_1. All plasmids were purified by two cycles of CsCl density gradient centrifugation.

In Vitro T_3 Binding Assays

Human *erb*Aα_1, -α_2, and -β cDNAs were also subcloned into pGEM vectors (Promega Biotec, Madison, WI) for *in vitro* transcription using T7 polymerase. Capped mRNA transcripts were used to program rabbit reticulocyte lysates according to the protocol of the supplier (Promega Biotec). ^{125}I-Labeled T_3 binding to the translated products was measured as described previously (31), using 0.10 nM [^{125}I]T_3 in the absence or presence of 1 mM unlabeled T_3 as a competitor for saturable binding sites.

Cell Culture and Transient Expression Assays

JEG-3 cells (HTB 36, American Type Culture Collection, Rockville, MD) were grown in Optimem media (Bethesda Research Laboratories-Gibco, Grand Island, NY) containing 2% (vol/vol) fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). In preparation for transient expression assays, large scale growups of JEG-3 cells were trypsinized, pooled, and plated onto 60-mm dishes in equal aliquots at a density of about 2×10^6 cells/plate. Eighteen hours before transfection, the freshly plated cells were grown in medium containing 2% charcoal-stripped fetal calf serum (to deplete it of T_3) and remained in this medium throughout the experiment. Transfections were performed in triplicate, using the calcium phosphate procedure (32). Transfected plasmids (15 μg) included 5 μg reporter plasmid (TreTKCAT or TSHαCAT), 5 μg pTKGH (a plasmid in which human GH expression is driven by the TK promoter) (33), and 0.2–5 μg thyroid hormone receptor expression vector with the addition of CDM8 plasmid as necessary to maintain a constant amount of DNA in each transfection. After 16-h exposure to the calcium phosphate/DNA precipitate, medium was removed, and 3 ml fresh serum-stripped medium with or without 5 nM T_3 was added. The transfected cells were incubated for an additional 48 h before harvest for CAT enzyme assays.

CAT activity was measured by quantitating the acetylation of [^{14}C]chloramphenicol (34). Cellular extracts were diluted to maintain enzyme activities within the linear range of the assay. CAT activity is defined as the percent conversion of chloramphenicol to acetylated derivatives per μl extract/h. The variability in CAT activity in triplicate transfections was typically less than 10%. Measurement of secreted GH derived from the cotransfected pTKGH did not reduce experimental variability among triplicate transfections, and in some instances, this additional measurement resulted in increased variability. Furthermore, we did not observe a consistent alteration in the level of expressed GH in the absence or presence of T_3 treatment, so that fold induction or repression effects were unaffected by correcting for GH secretion. For these reasons, CAT assay values were not corrected for coexpressed GH. In contrast to the relatively low variability among triplicate transfections, the CAT activity measured for a given construction in separate experiments varies 2- to 3-fold, due primarily to differences in transfection efficiency. For this reason, the represented data are all derived from a single group of transfections. Each experiment was, however, repeated at least three times with similar results.

Receptor-DNA Binding Assays

Receptor interactions with DNA were analyzed as described previously (16), except that extracts were prepared from COS-1 cells that had been transfected with CDMα_1, -α_2, or -β expression vectors. COS-1 cells (CRL 1650, American Type Culture Collection) were transferred to Optimem with 2% serum-stripped fetal bovine serum before transfection by the DEAE-dextran method (35). After 36 h, cells were harvested, and extracts were prepared, as described by Damm *et al.* (21). Receptor binding to DNA fragments was assessed using gel mobility shift assays (16, 17). The DNA-receptor complexes were labeled using either ^{32}P-labeled DNA (50 pmol) or receptors labeled with [^{125}I]T_3, as described previously (16, 17). A palindromic TRE sequence, TREp (5′-aaggggatccTCAGGTCATGACCTGAggatcccctt-3′), was used as a known high affinity binding site (17) and corresponds to the TRE sequence in TreTKCAT. The CREB-protein-binding site (5′-aaggggatccGTAAAAATTGACGTCATGGTAATTACACCAAGTACCCTTCAagatcttcct-3′) (29) derived from the TSH α promoter (−132 and −92 bp) was used as a control sequence that does not bind T_3 receptors (16).

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REFERENCES

1. Evans RM 1988 The steroid and thyroid hormone receptor superfamily. Science 240:889–895
2. Samuels HH, Forman BM, Horowitz ZD, Ye ZS 1988 Regulation of gene expression by thyroid hormone. J Clin Invest 81:957–967

3. Beato M 1989 Gene regulation by steroid hormones. Cell 56:335–344
4. Sap J, Munoz A, Damm K, Goldberg Y, Ghysdael J, Leutz A, Beug H, Vennstrom B 1986 The c-*erb*-A protein is a high-affinity receptor for thyroid hormone. Nature 324:635–640
5. Weinberger C, Thompson CC, Ong ES, Lebo R, Gruol DJ, Evans RM 1986 The c-*erb*-A gene encodes a thyroid hormone receptor. Nature 324:641–646
6. Thompson CC, Weinberger C, Lebo R, Evans RM 1987 Identification of a novel thyroid hormone receptor expressed in the mammalian central nervous system. Science 237:1610–1614
7. Benbrook D, Pfahl M 1987 A novel thyroid hormone receptor encoded by a cDNA clone from a human testis library. Science 238:788–791
8. Mitsuhashi T, Tennyson GE, Nikodem VM 1988 Alternative splicing generates messages encoding rat c-*erb* A proteins that do not bind thyroid hormone. Proc Natl Acad Sci USA 85:5804–5808
9. Nakai A, Seino S, Sakurai A, Szilak I, Bell GI, DeGroot LJ 1988 Characterization of a thyroid hormone receptor expressed in human kidney and other tissues. Proc Natl Acad Sci USA 85:2781–2785
10. Izumo S, Mahdavi V 1988 Thyroid hormone receptor α isoforms generated by alternative splicing differentially activate myosin HC gene transcription. Nature 334:539–542
11. Scheuler PA, Schwartz HL, Strait KA, Mariash CN, Oppenheimer JH 1990 Binding of 3,5,3′-triiodothyronine (T_3) and its analogs to the *in vitro* translational products of c-*erb* A protooncogenes: differences in the affinity of the α- and β-forms for the acetic acid analog and failure of the human testis and kidney α-2 products to bind T_3. Mol Endocrinol 4:227–234
12. Thompson CC, Evans RM 1989 *Trans*-activation by thyroid hormone receptors: functional parallels with steroid hormone receptors. Proc Natl Acad Sci USA 86:3494–3498
13. Koenig RJ, Warne RL, Brent GA, Harney JW, Larsen PR, Moore DD 1988 Isolation of a cDNA clone encoding a biologically active thyroid hormone receptor. Proc Natl Acad Sci USA 85:5031–5035
14. Koenig RJ, Lazar MA, Hodin RA, Brent GA, Larsen PR, Chin WW, Moore DD 1989 Inhibition of thyroid hormone action by a non-hormone binding c-*erb*A protein generated by alternative mRNA splicing. Nature 337:659–661
15. Lazar MA, Hodin RA, Chin WW 1989 Human carboxyl-terminal variant of α-type c-*erb* A inhibits *trans*-activation by thyroid hormone receptors without binding thyroid hormone. Proc Natl Acad Sci USA 86:7771–7774
16. Chatterjee VKK, Lee JK, Rentoumis A, Jameson JL 1989 Negative regulation of the thyroid-stimulating hormone α gene by thyroid hormone: receptor interaction adjacent to the TATA box. Proc Natl Acad Sci USA 86:9114–9118
17. Glass CK, Holloway JM, Devary OV, Rosenfeld MG 1988 The thyroid hormone receptor binds with opposite transcriptional effects to a common sequence motif in thyroid hormone and estrogen response elements. Cell 54:313–323
18. Lazar MA, Hodin RA, Darling DD, Chin WW 1988 Identification of a rat c-*erb* Aα-related protein which binds deoxyribonucleic acid but does not bind thyroid hormone. Mol Endocrinol 2:893–901
19. Seed B, Aruffo A 1987 Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. Proc Natl Acad Sci USA 84:3365–3369
20. Brent GA, Larsen PR, Harney JW, Koenig RJ, Moore DD 1989 Functional characterization of the rat growth hormone promoter elements required for induction by thyroid hormone with and without a co-transfected β type thyroid hormone receptor. J Biol Chem 264:178–182
21. Damm K, Thompson CC, Evans RM 1989 Protein encoded by v-*erb* A functions as a thyroid-hormone receptor antagonist. Nature 339:593–597
22. Sap J, Munoz A, Schmitt J, Stunnenberg H, Vennstrom B 1989 Repression of transcription mediated at a thyroid hormone response element by the v-*erb*-A oncogene product. Nature 340:242–244
23. Glass CK, Lipkin SM, Devary OV, Rosenfeld MG 1989 Positive and negative regulation of gene transcription by a retinoic acid-thyroid hormone receptor heterodimer. Cell 59:697–708
24. Forman BM, Yang C, Au M, Casanova J, Ghysdael J, Samuels HH 1989 A domain containing leucine-zipper-like motifs mediate novel *in vivo* interactions between the thyroid hormone and retinoic acid receptors. Mol Endocrinol 3:1610–1626
25. Lewin B 1990 Commitment and activation at Pol II promoters: a tail of protein-protein interactions. Cell 61:1161–1164
26. Murray MB, Towle HC 1989 Identification of nuclear factors that enhance binding of the thyroid hormone receptor to a thyroid hormone response element. Mol Endocrinol 3:1434–1442
27. Burnside J, Darling DS, Chin WW 1990 A nuclear factor that enhances binding of thyroid hormone response elements. J Biol Chem 265:2500–2504
28. Sakurai A, Nakai A, DeGroot LJ 1989 Expression of three forms of thyroid hormone receptor in human tissues. Mol Endocrinol 3:392–399
29. Jameson JL, Jaffe RC, Deutsch CA, Habener JF 1988 The gonadotropin α-gene contains multiple protein binding domains that interact to modulate basal and cAMP-responsive transcription. J Biol Chem 263:9879–9886
30. Nakai A, Sakurai A, Bell GI, DeGroot LJ 1988 Characterization of a third human thyroid hormone receptor coexpressed with other thyroid hormone receptors in several tissues. Mol Endocrinol 2:1087–1092
31. Inoue A, Yamakawa J, Yukioka M, Morisawa S 1983 Filter-binding assay procedure for thyroid hormone receptors. Anal Biochem 134:176–183
32. Graham FL, Van der Eb AJ 1973 A new technique for the assay of infectivity of human adenovirus DNA. Virology 52:456–467
33. Selden RF, Howie KB, Rowe ME, Goodman HM, Moore DD 1986 Human growth hormone as a reporter gene in regulation studies employing transient gene expression. Mol Cell Biol 6:3173–3179
34. Gorman CM, Moffat LF, Howard BH 1982 Recombinant genomes which express chloramphenicol acetlytransferase in mammalian cells. Mol Cell Biol 2:1044–1051
35. Deans RJ, Denis KA, Taylor A, Wall K 1984 Expression of an immunoglobin heavy chain gene transfected into lymphocytes. Proc Natl Acad Sci USA 81:1292–1296