Thyroid Hormone Receptor Mediates Transcriptional Activation and Repression of Different Promoters *in Vitro*

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The thyroid hormone receptor (TR) has the dual ability to activate or repress transcription of specific genes. A cell-free transcription system was used to study the effects of TR on transcription by positively (TREpMLP) and negatively (TSHα) regulated promoters. Receptor-deficient HeLa cell extracts were complemented with baculovirus-produced TR. TR stimulated transcription from the TREpMLP promoter by 3-fold, and *trans*-activation did not require hormone. Transcriptional stimulation by TR required the presence of the TRE sequence and was diminished by the addition of competitor TRE binding sites. Baculovirus-produced TR repressed transcription *in vitro* from the TSHα promoter by 30–50%, also in a hormone-independent manner. Transcription from a control adenovirus 2 major late promoter was unaffected by added TR. Receptor-specific antisera and competition with TRE binding sites impaired TR-mediated repression of the TSHα promoter. Unlike transcriptional stimulation, which was optimal when TR and HeLa extracts were added concomitantly, transcriptional repression by the TR was most effective when the receptor was preincubated with the α-promoter, suggesting that receptor binding to the promoter may block access of other proteins to cause transcriptional repression. These results indicate that baculovirus-expressed TR mediates transcriptional activation and repression in a promoter-specific manner *in vitro*. This system provides a valuable model for examining transcriptional control by the TR. (Molecular Endocrinology 6: 815–825, 1992)

INTRODUCTION

Thyroid hormone receptors (TRs) regulate a wide variety of developmental and metabolic processes by causing alterations in levels of gene expression (1, 2). A fundamental feature of TR action is the dual ability to cause transcriptional activation of some genes, but repression of other genes. Like other members of the nuclear receptor superfamily, transcriptional regulation by the TR is achieved by sequence-specific DNA binding to cognate response elements in target genes (3, 4).

After the cloning of nuclear receptor cDNAs, two major experimental approaches have been used to study transcriptional regulation by the receptors. In one approach, promoter sequences from hormone-responsive reporter genes have been linked to reporter genes to examine transcriptional regulation by coexpressed receptors in transient gene expression assays. This strategy has been valuable for defining the hormone response elements in target gene promoters and for characterizing functional domains within the receptors. Thyroid hormone-responsive sequences have been identified in multiple different positively regulated genes, allowing derivation of a consensus positive thyroid hormone response element (pTRE) (5, 6). The pTRE consists of the hexameric half-site, AGGTCA, arranged either as an inverted or direct repeat, generally located several hundred base pairs (bp) upstream of the transcriptional start site (7). Negative TREs (nTREs) have been less clearly defined than positive TREs. However, putative nTREs have been identified in the TSH α (8, 9) and β (10–12a) genes. In contrast to the positively regulated genes, receptor binding sites in the TSH α and β genes reside in the proximal region of the promoter, near the transcriptional start site. Based upon transient expression assays in receptor-deficient cells, it has been shown that cotransfected TR regulates transcriptional responses of both positive and negative reporter genes (9, 13).

Cell-free transcription assays have also been used to study mechanisms of transcriptional control by nuclear receptors. These assays have employed similar hormone-responsive reporter genes, and transcription has been studied either by supplementing cellular extracts

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containing basal transcription factors with nuclear receptors or by using extracts from cells that express large numbers of a specific receptor. Thus far, cell-free transcription reactions have been used for studies of positive regulation by the progesterone (14–16), glucocorticoid (17–20), and estrogen receptors (21, 22). These studies have confirmed that the nuclear receptors function as transcription factors and have allowed studies of transcriptional control by receptors to be examined in mechanistic terms. In the case of transcription mediated by the progesterone receptor (PR), there is evidence that the receptor functions in part by stabilizing the formation of a preinitiation complex (15). There is also evidence that nuclear receptors can activate transcription by altering chromatin structure to facilitate the binding of other transcription factors such as nuclear factor-1 (20, 23) or octamer transcription factor-1 (18) to DNA. With the further identification and purification of basal transcription factors as well as nuclear receptors, transcription reactions *in vitro* will provide a valuable approach for examining the kinetics of formation of the transcription complex and cofactors that are required for transcriptional control by nuclear receptors.

We recently described expression of the human TRβ receptor in a baculovirus expression system (24). With the availability of the large quantities of TR produced in this system, it is now feasible to reconstitute transcription reactions *in vitro* using the TR and thyroid hormone-regulated DNA templates. In this report, we sought to determine whether the baculovirus-expressed TR was transcriptionally active and to define experimental conditions that would allow studies of both positively and negatively regulated genes.

RESULTS AND DISCUSSION

Preparation of Human TRβ in a Baculovirus Expression System

In order to study the influence of the TR on transcription *in vitro*, the β-isoform of the human TRβ (hTRβ) was produced in large amounts using the baculovirus expression system (24). The characteristics of the receptor preparation used for these studies are shown in Fig. 1. TRβ constitutes about 10% of the total protein in the infected Sf9 cells. After extraction in high salt, TRβ represented approximately 50% of the protein (Fig. 1A) and was used without further purification. The TRβ in this and other preparations binds T_3 with physiological affinity (24), and T_3 binding was used to estimate the amount of active receptor in the preparation (~10–20% of expressed receptor binds T_3).

Gel mobility shift studies were used to assess the specificity of DNA binding by the receptor and to determine whether T_3 was required for receptor binding to the palindromic TRE (TREp). Extracts from cells infected with the recombinant baculovirus containing TRβ produced a major receptor-DNA complex when incubated with radiolabeled TREp (Fig. 1B). This complex was eliminated by the addition of 40-fold excess of unlabeled competitor TREp. Receptor-DNA complexes were not seen when receptor-containing extracts were incubated with other DNA sequences, including a cAMP response element from the α-promoter (CRE), or either of two elements from the Mullerian Inhibitory Substance (MIS) promoter, MISp or MISh. Control extracts from Sf9 cells infected with the wild type baculovirus did not produce a DNA-receptor complex with TREp (Fig. 1C, lane 1). The mobility of the TRβ-TREp complex was supershifted by addition of the TRβ-specific antibody (Fig. 1C, lanes 3 and 6) but not by the TRα2-specific antibody (Fig. 1C, lanes 4 and 7), confirming that these complexes contain receptor. Addition of T_3 was not required for receptor binding to TREp and did not alter the amount of receptor-DNA complex (Fig. 1C, lanes 5–7). The TRβ extracts were also shown to produce DNaseI footprints over TREp in a reporter gene construct (TRETKCAT) and over the nTRE (−22 to −7 bp) in the TSHα promoter (Madison, L. D., unpublished data). Taken together, these results indicate that a fraction of the baculovirus-expressed TRβ is functional as assessed by binding of its ligand (T_3) with high affinity, recognition by receptor-specific antibody, and interaction with both positive and negative TREs. Receptor binding to DNA appears to be lower than expected based on T_3 binding. This may reflect the inability of the gel shift assay to measure DNA binding quantitatively. Alternatively, the absence of accessory proteins important for receptor binding to DNA or the lack of important posttranslational modifications may account for the relatively low DNA binding activity (25–28).

Cell-Free Run-Off Transcription of the α-Promoter

The α-gene promoter contains a well characterized enhancer that consists of an upstream regulatory element (URE) between −180 and −151 bp and two copies of a CRE (−146/−111 bp), which binds CRE binding protein and mediates cAMP stimulation of α-transcription (Fig. 2A, *top*) (29, 30). In addition, the α-promoter contains a nTRE that has been localized in the proximal region of the promoter, immediately adjacent to the TATA box (9). Cell-free transcription of the α-promoter was examined initially using a run-off transcription assay. Templates for run-off transcription included −846αCAT (pα), and truncated constructs lacking upstream pα sequences, but including the URE and CRE regions (−195α) or CRE region alone (−156α) linked to a chloramphenicol acetyltransferase (CAT) gene fragment linearized with *Eco*R1 (Fig. 2A). These constructs create a template with a predicted run-off transcription product of 294 nucleotides (nt) (31). A control adenovirus 2 major late promoter (MLP) plasmid produces a predicted run-off transcript of 220 nt. HeLa whole cell extracts were preincubated with template DNA for 30 min before the addition of NTPs to initiate transcription. Correctly initiated transcripts from the pα constructs and the MLP are evident after run-off transcription. (Fig.

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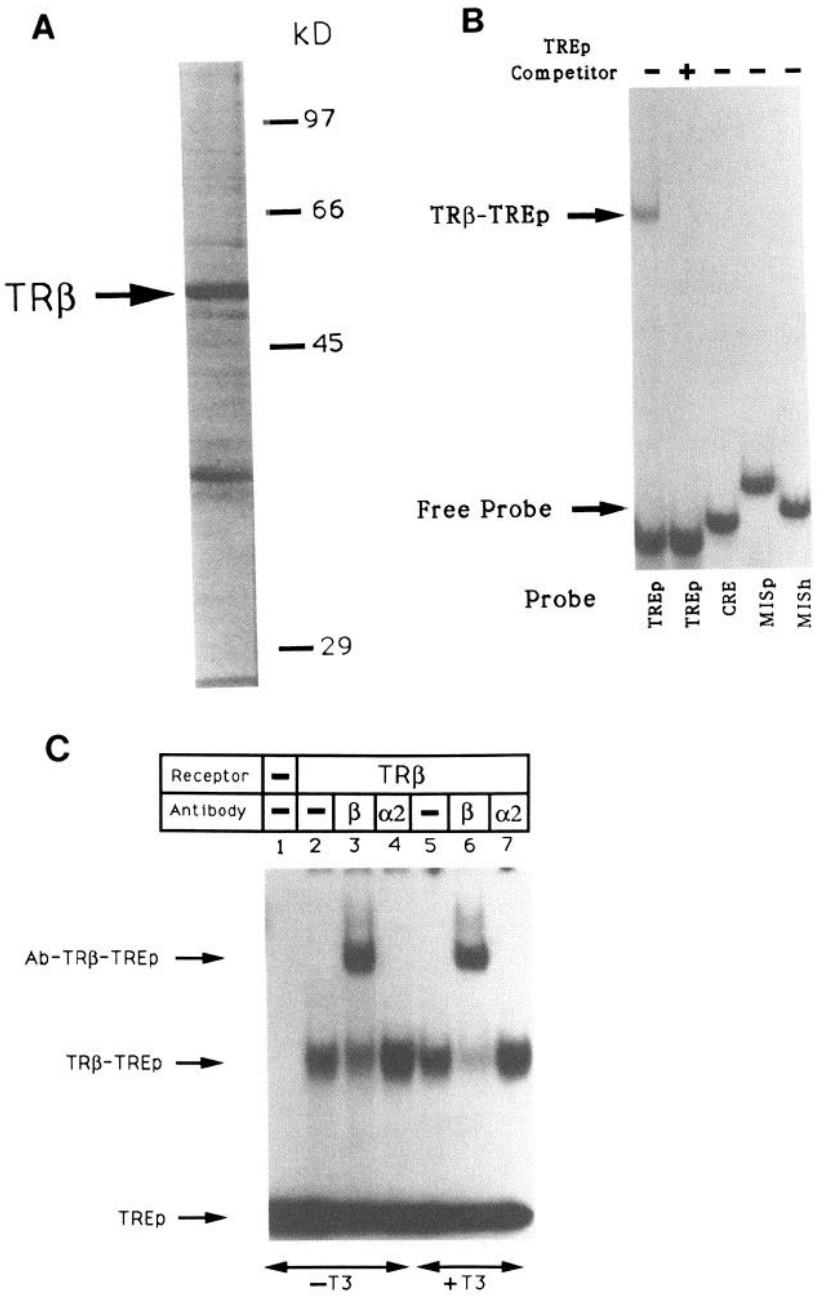

Fig. 1. A, Baculovirus-Expressed TRβ

Coomassie-blue stained sodium dodecyl sulfate-polyacrylamide gel of TRβ-containing extract (30 μg total protein) prepared by high salt extraction (24). The TRβ band is indicated with an *arrow*, and mol wt markers are indicated at the *right*. B, Binding of Sf9 extracts containing TRβ is specific for TRs. Extracts from Sf9 cells containing overexpressed TRβ (5 pmol) were incubated with ^{32}P-labeled TREp oligonucleotide (50 fmol) in the absence (−) or presence (+) of competitor TREp (2 pmol). Additional labeled DNA fragments included a consensus CRE and two different protein binding elements in the MIS promoter, MISp, MISh. The position of the specific TRβ-TREp complex is indicated by an *arrow*. C, Binding of TRβ to TREp is ligand independent. Extracts from Sf9 cells infected with wild type (lane 1) or TRβ-containing recombinant baculovirus (lanes 2–7) were incubated without (lanes 1–4) or with T_3 (lanes 5–7) before binding to ^{32}P-labeled TREp. Anti-TRβ (lanes 3 and 6) or anti-TRα2 (lanes 4 and 7) antibodies were added to the indicated reactions. Receptor-DNA (TRβ-TREp) and antibody supershifted (Ab-TRβ-TREp) complexes are indicated by *arrows*.

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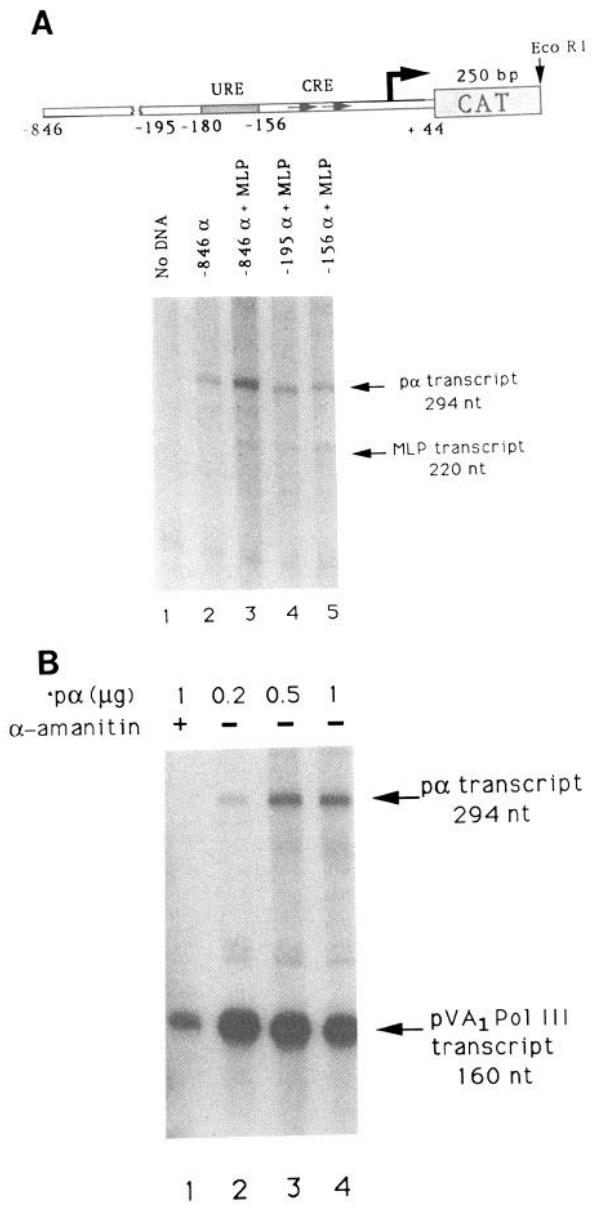

Fig. 2. A, Run-Off Transcription from the α-Promoter

The α-promoter is illustrated at the *top* of the figure, indicating the location of an upstream regulatory element (URE, □), and two copies of a CRE (*arrows*). The site of transcriptional initiation and the position of an *Eco*R1 site used to linearize the plasmid are indicated. Lane 1, no DNA; lane 2, −846αCAT without MLPCAT; lane 3, −846αCAT with MLPCAT; lane 4 −195αCAT with MLPCAT; lane 5, −156αCAT with MLPCAT. B, Run-off transcription of the α-promoter is α-amanitin sensitive. Run-off transcription reactions were performed using the indicated amounts (0.2–1.0 μg) of linearized pα plasmid and a constant amount of pVA$_1$ plasmid (1 μg) in the presence (+) or absence (−) of α-amanitin (10 μg/ml). The 294-nt pα transcript and the 160-nt pVA$_1$ *Pol*III transcript are indicated by *arrows*.

2A, lanes 2–5). The transcriptional activity of the truncated pα constructs was similar to the −846α construct when correction is made for the amount of MLP transcript. Deletion of the CRE enhancer elements (−100αCAT) reduced the activity of the promoter (data not shown) (32).

The specificity of *Pol*II-dependent pα transcription was assessed by examining sensitivity to inhibition by α-amanitin. Production of the pα transcript was completely inhibited by α-amanitin (10 μg/ml) (Fig. 2B, lane 1 *vs.* 4), whereas the 160-nt pVA$_1$ *Pol*III transcript was only partially inhibited by α-amanitin treatment. These results confirm that transcription of the α promoter is mediated by RNA *Pol*II.

Construction of Positively and Negatively Regulated DNA Templates for Transcription *in Vitro*

To facilitate the analysis of TR regulation of cell-free transcription reactions, additional studies were conducted using promoters linked to G-free cassettes. The templates for transcription reactions *in vitro* were constructed using promoter sequences that contain T_3 response elements that have been characterized previously in transient expression assays (Fig. 3) (13, 33). For construction of a positively regulated promoter, two copies of the palindromic positive TRE were inserted upstream of the adenovirus MLP, a strong basal promoter that has been studied extensively with cell-free transcription assays (34). In transient expression assays, this optimized positive TRE is differentially regulated in the absence and presence of T_3. In the absence of hormone, cotransfected TR suppresses the basal activity of pTRE-linked reporter genes; addition of T_3 relieves this basal inhibition and causes additional transcriptional activation (13, 35, 36). The negatively regulated promoter pα was constructed using −713 to +44 bp of the α-subunit gene. The pα template was de-

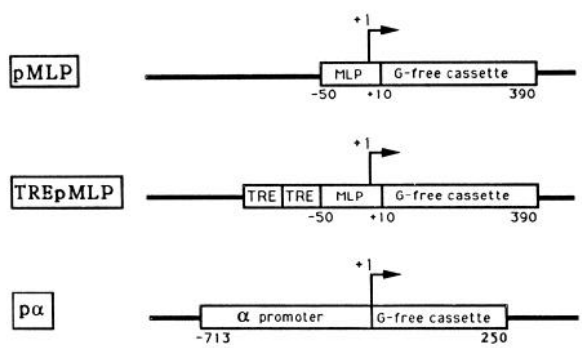

Fig. 3. Schematic Representation of Control and TRE-Containing Templates for Transcription *in Vitro*

The control template, pMLP, contains a 390-bp G-free cassette (34). Two copies of TREp were subcloned upstream of pMLP to create a positively regulated template, TREpMLP. The TSH α-promoter construct was ligated to a 250-bp G-free cassette to generate the negatively regulated template, pα.

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signed to retain basal enhancer sequences and provides a level of basal transcription that is high enough to allow measurement of transcriptional repression. In transient expression assays, the cotransfected TR does not affect basal expression of the α-promoter, but addition of T_3 causes a 50–80% inhibition of promoter activity (13, 37).

Transcriptional Stimulation *in Vitro* by TR

Because several members of the nuclear receptor superfamily have been demonstrated to stimulate transcription, we next characterized the activity of the baculovirus-expressed TRβ using the positively regulated promoter, TREpMLP. The conditions for transcription *in vitro* were optimized by titrating the amounts of supercoiled DNA and HeLa extract. With different preparations of plasmid and HeLa extracts, maximal levels of transcription were obtained using 0.25–0.50 pmol DNA and 50–100 μg protein in the whole cell extracts (data not shown). Because both the control MLP and the TREpMLP templates each generated 390 nt T_1 RNase-resistant transcripts, replicate reactions were performed in parallel with each template. Initially, the TRβ extracts were added to the transcription reactions concomitant with the HeLa extract and nucleotides (Fig. 4A). Addition of control Sf9 extracts from cells infected with the wild type baculovirus did not alter the level of basal transcription (data not shown), and all reactions contained an equivalent amount of control or TRβ-containing Sf9 extract protein. Addition of TRβ stimulated transcription from TREpMLP in a dose-dependent manner (Fig. 4A, *upper panel*) but did not alter transcription from the control MLP template (Fig. 4A, *lower panel*). Maximal transactivation (3- to 5-fold) of TREpMLP occurred with 1 pmol receptor. To confirm that transcriptional stimulation by TRβ was mediated by specific binding to the TRE, 10-fold excess TREp oligonucleotide was added to the reactions (Fig. 4A, lanes 4 and 8). The competitor TREp eliminated *trans*-activation by TRβ from the TREpMLP template, but the level of transcription from pMLP was unaffected by competitor oligonucleotide. In other experiments, a competitor oligonucleotide that does not bind TRβ (TSHα sequence −132/−92) (9) had no effect on *trans*-activation. *Trans*-activation by TRβ occurred both in the absence and the presence of T_3. Although the degree of TR-stimulated transcription was increased minimally by T_3 in some reactions, this effect was not seen consistently, and *trans*-activation was ligand independent. The results of three different experiments performed under these reaction conditions are summarized in Table 1. Using 1 pmol TRβ in the presence of T_3, the mean degree of *trans*-activation ranged from 2.3- to 3.5-fold. These data indicate that the baculovirus-expressed TRβ is transcriptionally active and that transcriptional activation requires binding to the TREp sequence.

In most transient expression assays, TR regulation of transcription is dependent on T_3. It was therefore surprising to find that T_3 was not required for transactivation *in vitro*. There are, however, several recent examples of constitutive activity of the TR in some tissues (38) and cell types (39), as well as in yeast expression systems (40). Hormone-independent activity has also been seen with highly purified preparations of progesterone receptor (15) and with baculovirus-expressed glucocorticoid receptor (19) in cell-free transcription reactions. However, other receptor preparations do exhibit hormone-dependent transcriptional activation *in vitro* (14, 21). The mechanisms that allow constitutive *vs.* hormone-regulated receptor action in these different experimental systems are unknown. Hormone binding may be required to allow posttranslational modifications to occur (41), or the ligand may induce a conformational change that alters receptor interactions with accessory proteins (14). Under some experimental conditions, the receptor may be able to bypass these regulatory steps to function in a constitutive manner.

The conditions of the cell-free transcription reaction allow the sequence in which the different components are added to be varied. Preincubation of TRβ with TREpMLP before the addition of HeLa extract resulted in less transcriptional activation by the receptor (data not shown). When the TREpMLP template was preincubated with HeLa extract to allow the formation of a preinitiation complex before addition of receptor, the subsequent addition of TRβ suppressed the level of basal transcription (Fig. 4B). Addition of T_3 relieved the basal suppression of TREpMLP but did not increase transcription to the level that was seen when receptor and HeLa extract were added concomitantly. Under these conditions, the receptor did not affect transcription from the MLP promoter, which lacks the TRE sequence. These results indicate that the order in which transcription factors are allowed to interact with template DNA can have significant effects on the properties of TR-mediated transcription. Diminished transcriptional stimulation after the formation of a preinitiation complex has also been observed with the progesterone (42) and glucocorticoid (17) receptors. It is possible that with some promoters, the preincubation step obviates the need for the receptor. Alternatively, preincubation with the HeLa extracts may prevent effective binding of the receptors to their hormone response elements. In the case of the TR, the mechanism for basal suppression in the absence of T_3 is not known, but it may be caused by receptor interference with the preinitiation complex. These results *in vitro* potentially share a mechanistic basis with the phenomenon of basal suppression that has been seen in transient expression assays in the absence of T_3 (35, 36).

In some studies, as much as 30-fold *trans*-activation by nuclear receptor superfamily members has been observed (15, 19, 22). By comparison, the degree of *trans*-activation by TRβ in this system was modest (2- to 4-fold), but consistently observed with different preparations of extracts. The lower degree of stimulation may be an inherent property of the TR. More likely, it

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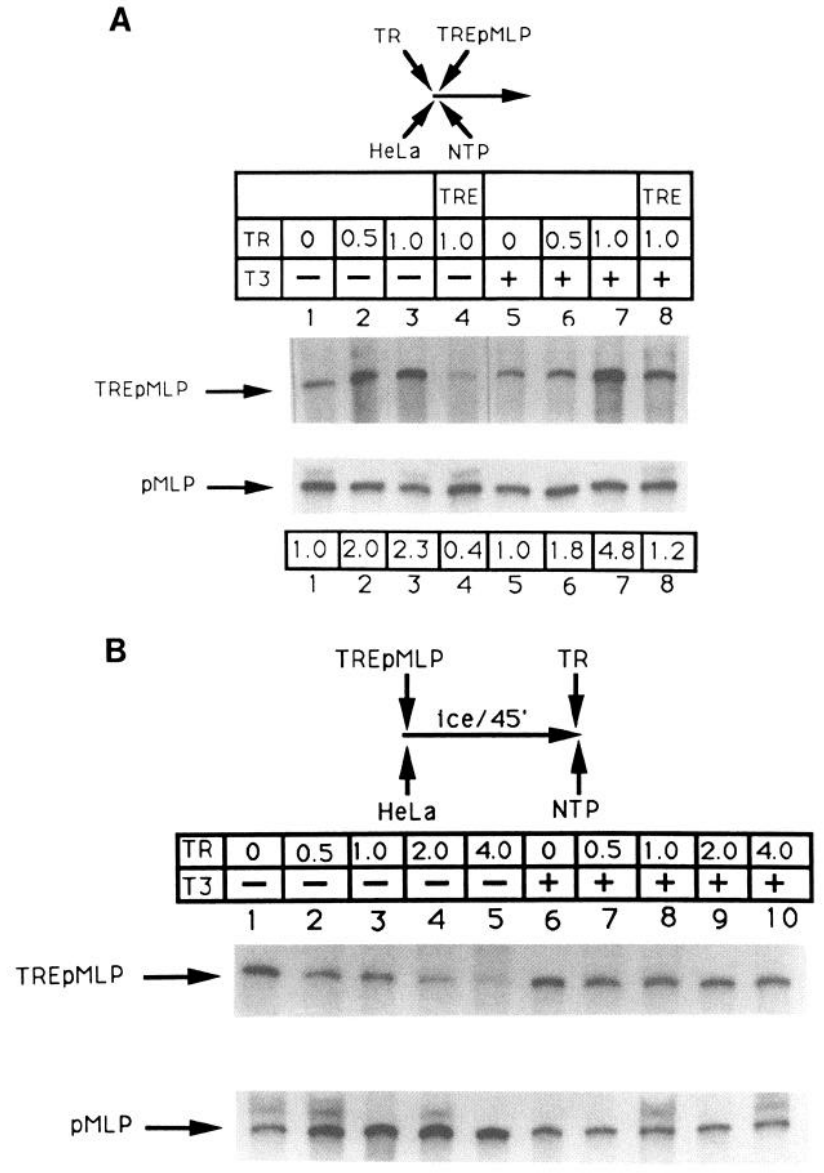

Fig. 4. A, TRβ Stimulates Cell-Free Transcription of a Promoter Linked to TREp

Reactions contain template DNA and control baculovirus extract (lanes 1 and 5) or control extracts supplemented with TRβ as indicated in picomoles at the *top* of the figure. Control or TRβ extract was preincubated without T_3 (lanes 1–4) or with T_3 (lanes 5–8). Competitor TREp was added to some reactions (lanes 4 and 8). Transcription reactions were started by the simultaneous addition of template DNA, TRβ, HeLa extract, and NTPs. The TREpMLP and pMLP transcripts are indicated by *arrows* to the left. *Numbers* below the lanes represent the degree of stimulation as measured by densitometric analyses. B, Ligand-dependent reversal of basal inhibition by TRβ. TREpMLP (*upper panel*) or pMLP (*lower panel*) was coincubated with HeLa extract for 45 min on ice before addition of the indicated amounts of TRβ (without T_3, lanes 1–5; or with T_3, lanes 6–10) and NTPs to start transcription. Conditions were otherwise identical to reactions in A.

is possible that larger effects could be obtained with further modifications of the experimental parameters. The degree of TR stimulation of the TREpMLP template may have been limited to some degree by the relatively high level of basal expression from the MLP promoter. In addition, the nature and location of the TREs in relation to the MLP promoter could influence the degree of stimulation by TR. Another important variable that deserves further study is the source of the receptor preparation. Thus far, we have only examined the activity of baculovirus-expressed TR. Although this system provides large quantities of relatively pure receptor, it is unclear what fraction of the preparation is transcriptionally competent. Furthermore, because only 10–20% of the preparation binds T_3, it is difficult to quantitatively assess the role of ligand binding in transcriptional regulation. Synergistic interactions between receptors and other transcription factors that bind to specific promoter sequences have been well documented for the progesterone (18), glucocorticoid (20), and estrogen receptors

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Table 1. Summary of Transcriptional Activation of TREpMLP and Repression of TSHα by TRβ

Promoter	Exp 1	Exp 2	Exp 3
TREpMLP[a]			
+TR/−TR ratio	3.5 ± 0.9	2.3 ± 0.8	2.9 ± 0.5
	n = 3	n = 5	n = 3
TSHα[b]			
+TR/−TR ratio	0.70 ± 0.15	0.72 ± 0.09	0.53 ± 0.22
	n = 6	n = 4	n = 6

[a] Reaction conditions for transcriptional activation of the TREpMLP promoter by TRβ are as described in the legend to Fig. 4A. Baculovirus-expressed TRβ (1 pmol) was preincubated with T_3, and reactions were started by the simultaneous addition of template DNA, TRβ, HeLa extract, and NTPs. Results are shown as mean ± SD.

[b] Reaction conditions for transcriptional repression of the TSHα promoter by TRβ are as described in the legend to Fig. 5B. Baculovirus-expressed TRβ (5 pmol) was preincubated with T_3 and then incubated with TSHα template DNA for 30 min at room temperature before transcription was started by the addition of HeLa extract and NTPs. Results are shown as mean ± SD.

(22), and it is possible that similar interactions may be required for greater stimulation by the TR.

Transcriptional Repression *in Vitro* by TR

Having shown that TRβ could mediate *trans*-activation of a positively regulated template, transcriptional repression of a negatively regulated gene was examined using the pα template. Because the length of the transcript from the pα (250 nt) and the pMLP promoters (390-nt transcript) are different, the MLP template could be included in the reactions to serve as an internal control and as an index for RNA recovery. In some experiments using the pα template, a 263-nt transcript was also observed, presumably due to read-through transcription from an upstream start site, as a 13-bp G-free sequence is present in pα immediately adjacent to the G-free cassette.

Initially, conditions were used that had allowed basal suppression to occur in the setting of the TREpMLP template. The pα template was preincubated with HeLa extract before addition of TRβ, without or with T_3. Under these conditions, TRβ did not alter transcription from pα, and transcription from pMLP was unaffected (Fig. 5A). Thus, unlike the situation with TREpMLP, the addition of TR after the presumed formation of a preinitiation complex did not suppress basal transcription. The incubation strategy was then reversed to favor interactions of TRβ with the nTRE. pα was preincubated with control Sf9 extract or with TRβ, without or with T_3, before addition of HeLa extract and NTPs to start transcription. Under these conditions, TR caused dose-dependent transcriptional repression (Fig. 5B). Using the largest dose of TR (10-fold excess receptor over template), as much as 60–70% repression was observed, but transcription from the internal control (pMLP) was unaffected (Fig. 5B), indicating that inhibition was specific for the pα promoter. Repression of pα by TRβ was also T_3 independent. A summary of three different experiments examining TRβ-mediated repression of pα under these reaction conditions is shown in Table 1. Using 5 pM TRβ in the presence of T_3, the mean degree of repression ranged from 28–47%.

To determine whether the preformed interaction between pα and TRβ could be challenged with basal transcription factors, pα was preincubated with TRβ followed by addition of HeLa extract with further coincubation before the addition of NTPs to start transcription. In this paradigm, repression of pα by TRβ was still observed (Fig. 5C). To confirm that repression of pα was mediated by TRβ, a TRβ-specific antibody, which recognizes an epitope in the amino-terminal region of TRβ, was included and partially reverted TRβ-mediated repression (Fig. 5C). In other experiments, addition of a TREp oligonucleotide also blocked TRβ-mediated repression of pα (data not shown).

Because there are few well characterized models of genes that are negatively regulated by members of the nuclear receptor superfamily, previous studies have not examined transcriptional repression in cell-free systems. The α-promoter provides a particularly useful model system, as its regulation by thyroid hormone has been extensively characterized *in vivo* (43) as well as in transient expression studies (9, 13). It is important to distinguish TR-mediated transcriptional repression of the α-promoter from the basal suppression that is seen with the TREp-linked promoter. The experimental conditions under which these phenomena occur are very different. Preincubation with TRβ was required for transcriptional repression of the α-promoter but did not repress transcription from the TREpMLP template or the MLP promoter. On the other hand, basal inhibition of the TREp-linked promoter occurred only when TRβ was added after the formation of a preinitiation complex. Under these conditions, transcription of the α-promoter was unaffected. These findings suggest that TRβ interactions with positively and negatively regulated templates may be fundamentally different. Although interactions with DNA are required for TRβ regulation of both types of promoters (9), the nature of the TRE sequence, its location in the promoter, and the order in which adjacent transcription factors interact could each account for distinct types of transcriptional regulation.

These experiments demonstrate that a cell-free transcription system can be used to examine mechanisms of *trans*-activation as well as repression by nuclear hormone receptors. This is the only report to date of

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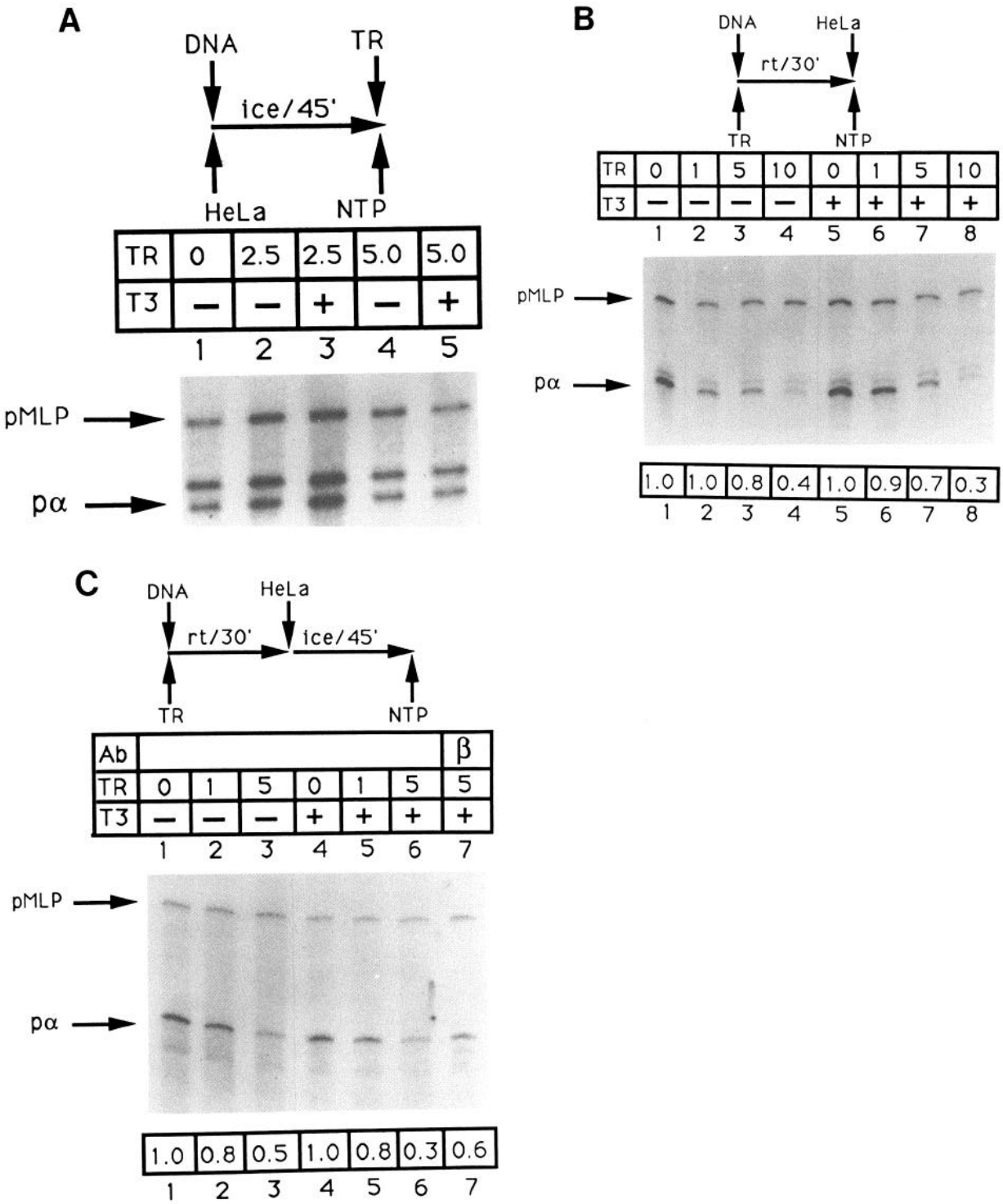

Fig. 5. Transcriptional Repression of the TSHα Promoter *in Vitro* by TRβ

A, Preincubation of template with HeLa extract prevents repression by TRβ. pMLP (internal control) and pα (nTRE containing α-promoter linked to G-free cassette) templates were incubated with HeLa extract for 45 min on ice. Reactions contained control extract (lane 1) or the indicated amounts of TRβ, without (lanes 2 and 4) or with (lanes 3 and 5) T_3. pMLP and pα transcripts are indicated with *arrows* to the left. B, Preincubation of template with TRβ causes transcriptional repression. Control extract (lanes 1 and 5) and indicated amounts of TRβ, without (lanes 2 and 3) or with (lanes 5 and 6) T_3, were preincubated with pMLP and pα for 30 min at room temperature. Transcription was started by addition of HeLa extract and NTPs, under conditions otherwise identical to those described above. The *numbers* below the lanes indicate the degree of repression as measured by densitometric analyses and normalized with respect to the pMLP internal control. C, Preincubated template-TRβ interaction is refractory to subsequent challenge by basal transcription factors. pMLP and pα, control extract (lanes 1 and 4), indicated amounts of TRβ, without (lanes 2 and 3) or with (lanes 5–7) T_3, and 1 μl (1:10 dilution) of anti-TRβ antibody (lane 7) were preincubated for 30 min at room temperature. HeLa extract was added and incubation continued for 45 min on ice before start of transcription by addition of NTPs. The degree of repression is indicated *below* the lanes.

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transcriptional repression *in vitro* by a member of the nuclear receptor family, although negative regulation has been observed for other classes of transcription factors. The homeodomain protein, *engrailed*, represses transcription *in vitro* by competition for TFIID binding to the TATA box. Like the situation with TR-mediated repression, *engrailed* caused inhibition only when added before TFIID. (44). Similarly, a cellular protein (LBP) binds to a site located downstream of the TATA box in the HIV-1 promoter and causes transcriptional repression when added to the promoter DNA before formation of the preinitiation complex (45). It is intriguing that the putative nTRE in the α-gene is located in a similar position, immediately adjacent to the TATA box. Interference by transcription factors bound to sites located at or near the transcription start site may provide a general mechanism for negative regulation of gene expression, but this model requires further study in view of the potential for proteins to interact at multiple steps in the process of transcription. As purified transcription factors become available, it will be useful to combine reconstitution experiments and kinetic analyses in cell-free transcription assays to better understand TR-mediated transcriptional activation and repression.

MATERIALS AND METHODS

Expression of TRβ

The baculovirus expression vector system was used to overexpress TRβ in insect (Sf9) cells, as described previously (24). The amount of expressed TRβ that was capable of binding to T_3 was estimated by [^{125}I]T_3 filter binding and Scatchard analyses (46).

Gel Shift Assays

Gel shift assays were performed as described previously (13). Oligonucleotides with the following sequences were used in the gel shift experiments: TREp (sense, 5′-gatcCTCAGGTCATGACCTGAC-3′; antisense, 3′-GAGTCCAGTACTGGACTGagct5-′); CRE (sense, 5′-gatcCAAAATTGACGTCATGGTAATTA-3′; antisense, 3′-GTTTTAACTGTACCATTAATctag-5′); MISp (sense, 5′-gatcTCCACCCTCAGGCACCAGGGGTGGACCCCCAGCCT-3′; antisense, 3′-AGGTGGGAGTCCGTGGTCCCCACCTGGGGGTCGGActag-3′); MISh (sense, 5′-gatcTAAGTCAATTAAACAGCCTCCCCCATGT-3′; antisense, ATTCAGTTAATTTGTCGGAGGGGGTACActag-5′). The CRE contains a palindromic CRE. The MIS sequences are protein binding elements from the MIS promoter, defined by gel-shift and footprinting assays (Haqq, C., unpublished data). Oligonucleotides were annealed and gel purified before radiolabeling with ^{32}P and Klenow enzyme. Oligonucleotides (0.05 pmol) were incubated with Sf9 cell extract containing approximately 5 pmol TRβ and subjected to nondenaturing polyacrylamide electrophoresis. Unlabeled competitor TREp (2 pmol) was included in some binding reactions. Polyclonal antibodies (1:10 dilution) specific for TRβ or TRα2 (47) were added to some of the reactions to cause a mobility shift of receptor-DNA complexes.

Run-Off Transcription Reactions

Whole cell extracts were prepared from receptor-deficient HeLa cells according to method of Manley *et al.* (48). Run-off transcription reactions were performed using various 5′-deletions of the α-promoter in CAT expression plasmids, linearized within the CAT gene using *Eco*R1. The control plasmid Ad2MLPCAT was linearized with the enzyme *Pvu*II. These constructs generated run-off transcription products of 294 and 220 nt, respectively. The RNA *Pol*III plasmid pVA1 was provided by Dr. M. Harter (Cleveland Clinic Foundation, Cleveland, OH). Run-off transcription reactions were performed in a buffer consisting of 20 mM HEPES-NaOH, pH 7.9, 5 mM $MgCl_2$, 60 mM KCl, 1.5 mM dithiothreitol, and 12% glycerol. Each reaction contained 30 μl extract (80 μg protein) in a 50-μl reaction. Plasmid DNA (1 μg) was preincubated with extract on ice for 60 min before initiating transcription by the addition of 0.5 mM NTPs containing [α-^{32}P]CTP and 5 mM creatine phosphate.

Transcription Reactions Using G-Free Cassettes

The plasmid pMLP (kindly provided by M. Sawadogo, University of Texas, Houston, TX) contains promoter sequences from the adenovirus 2 major late promoter between −50 to +10, linked to a 380-bp G-free cassette (Fig. 3) (34). TREpMLP was constructed by cloning two copies of a palindromic positive TRE (5, 6) into the *Bam*HI site located immediately upstream of the promoter in pMLP. Pα contains 713 bp 5′-flanking sequence of the human TSHα gene (9) linked to a G-free cassette (derived from pMLP) by blunt-end ligation into a *Pst*I site located at +1 relative to the transcription initiation site. Transcription reactions using the G-free-linked cassettes contained HeLa extract (80 μg protein) and 0.3 pmol (1 μg) DNA template in a reaction mixture (50 μl) consisting of 20 mM HEPES-NaOH, pH 7.9, 6 mM $MgCl_2$, 60 mM KCl, 2 mM dithiothreitol, 0.02 mM EDTA, 0.02 mM EGTA, 10% glycerol, 0.05 mM UTP, 0.5 mM rATP, 0.5 mM rCTP, 5 mM creatine phosphate, 20 U T_1 RNase, and 5 μCi [α-^{32}P]UTP (800 Ci/mmol). Control extracts from Sf9 cells infected with wild type baculovirus or extracts containing varying amounts of baculovirus-expressed TRβ (0–10 pmol) were added to the transcription reactions as indicated in the figure legends. In reactions containing T_3, receptor-containing extracts were preincubated with 20 nM T_3 overnight at 4 C. All reactions were supplemented with control Sf9 cell extract to maintain constant protein concentrations. Reactions were incubated at 30 C for 45 min followed by addition of 150 μl stop solution (0.1 mg/ml proteinase K, 50 mM Tris-HCl, pH 7.5, 50 mM EDTA, 0.5 M NaCl, and 5% sodium dodecyl sulfate) and incubation at 37 C for 15 min. RNA was isolated after three cycles of ethanol precipitation and subjected to electrophoresis through a 5% polyacrylamide 8 M urea gel at 40 mA constant current. Transcripts were visualized by autoradiography, and scanning densitometry was performed using a LKB (Piscataway, NJ) Ultroscan laser densitometer.

Acknowledgments

We are grateful to Y. Hwang for technical assistance, E. Macchia and L. DeGroot for TR antibodies, M. Sawadogo for G-less cassette constructs, I. Taylor and J. Hoeffler for baculovirus expression vectors, and P. Bradley and J. Zu Putlitz for advice regarding the baculovirus expression system. We thank our colleagues in the Jameson and R. Young labs (Whitehead Institute, Massachusetts Institute of Technology) for many helpful discussions.

Received December 18, 1991. Revision received February 24, 1992. Accepted March 9, 1992.

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This work was supported in part by PHS Grants DK-42144 and HD-28138.

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REFERENCES

1. Samuels HH, Forman BM, Horowitz ZD, Ye ZS 1989 Regulation of gene expression by thyroid hormone. Annu Rev Physiol 51:623–639
2. Lazar MA, Chin WW 1990 Nuclear thyroid hormone receptors. J Clin Invest 86:1777–1782
3. Evans RM 1988 The steroid and thyroid hormone receptor superfamily. Science 240:889–895
4. Beato M 1991 Transcriptional control by nuclear receptors. FASEB J 5:2044–2051
5. 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
6. Brent GA, Harney JW, Chen Y, Warne RL, Moore DD, Larsen PR 1989 Mutations in the rat growth hormone promoter which increase and decrease response to thyroid hormone define a consensus thyroid hormone response element. Mol Endocrinol 3:1996–2004
7. Naar AM, Boutin J-M, Lipkin SM, Yu VC, Holloway JM, Glass CK, Rosenfeld MG 1991 The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. Cell 65:1267–1279
8. Burnside J, Darling DS, Carr FE, Chin WW 1989 Thyroid hormone regulation of the rat glycoprotein hormone alpha-subunit gene promoter activity. J Biol Chem 264:6886–6891
9. Chatterjee VKK, Lee JK, Rentoumis A, Jameson JL 1989 Negative regulation of the thyroid-stimulating hormone alpha gene by thyroid hormone: receptor interaction adjacent to the TATA box. Proc Natl Acad Sci USA 86:9114–9118
10. Carr FE, Burnside J, Chin WW 1989 Thyroid hormones regulate rat thyrotropin-β gene promoter activity expressed in GH3 cells. Mol Endocrinol 3:709–716
11. Wondisford FE, Farr EA, Radovick S, Steinfelder HJ, Moates JM, McClaskey JH, Weintraub BD 1989 Thyroid hormone inhibition of human thyrotropin beta-subunit gene expression is mediated by a *cis*-acting element located in the first exon. J Biol Chem 264:14601–14604
12. Wood WM, Kao MY, Gordon DF, Ridgway EC 1989 Thyroid hormone regulates the mouse thyrotropin beta-subunit gene promoter in transfected primary thyrotropes. J Biol Chem 264:14840–14847

12a. Bodenner DL, Mroczynski MA, Weintraub BD, Radovick S, Wondisford FE 1991 A detailed functional and structural analysis of a major thyroid hormone inhibitory element in the human thyrotropin β-subunit gene. J Biol Chem 266:21666–21673

13. Rentoumis A, Chatterjee VKK, Madison LD, Datta S, Gallagher GD, DeGroot LJ, Jameson JL 1990 Negative and positive transcriptional regulation by thyroid hormone receptor isoforms. Mol Endocrinol 4:1522–1531
14. Bagchi MK, Tsai SY, Weigel NL, Tsai M, O'Malley BW 1990 Identification of a functional intermediate in receptor activation in progesterone-dependent cell-free transcription. Nature 345:5129–5134
15. Klein-Hitpass L, Tsai SY, Weigel NL, Allan GF, Riley D, Rodriguez R, Schrader WT, Tsai M, O'Malley BW 1990 The progesterone receptor stimulates cell-free transcription by enhancing the formation of a stable preinitiation complex. Cell 60:247–257
16. Bagchi MK, Tsai SY, Weigel NL, Tsai MJ, O'Malley BW 1990 Regulation of *in vitro* transcription by progesterone receptor. Characterization and kinetic studies. J Biol Chem 265:5129–5134
17. Freedman LP, Yoshinaga SK, Vanderbilt JN, Yamamoto KR 1989 *In vitro* transcription enhancement by purified derivatives of the glucocorticoid receptor. Science 245:298–301
18. Bruggemeier U, Kalff M, Franke S, Scheidereit C, Beato M 1991 Ubiquitous transcription factor OTF-1 mediates induction of the MMTV promoter through synergistic interaction with hormone receptors. Cell 64:565–572
19. Tsai S-Y, Srinivasan G, Allan GF, Thompson EB, O'Malley BW, Tsai M-J 1990 Recombinant human glucocorticoid receptor induces transcription of hormone response genes *in vitro*. J Biol Chem 265:17055–17061
20. Allan GF, Ing NH, Tsai SY, Srinivasan G, Weigel NL, Thompson EB, Tsai M-J, O'Malley BW 1991 Synergism between steroid response and promoter elements during cell-free transcription. J Biol Chem 266:5905–5910
21. Corthesy B, Hipskind R, Theulaz I, Wahli W 1988 Estrogen-dependent *in vitro* transcription from the vitellogenin promoter in liver nuclear extracts. Science 239:1137–1139
22. Corthesy B, Claret FX, Wahli W 1990 Estrogen receptor level determines sex-specific *in vitro* transcription from the *Xenopus* vitellogenin promoter. Proc Natl Acad Sci USA 87:7878–7882
23. Corthesy B, Corthesy TI, Cardinaux JR, Wahli W 1991 A liver protein fraction regulating hormone-dependent *in vitro* transcription from the vitellogenin genes induces their expression in *Xenopus* oocytes. Mol Endocrinol 5:159–169
24. Zu Putlitz J, Datta S, Madison LD, Macchia E, Jameson JL 1991 Human thyroid hormone β1 receptor produced by recombinant baculovirus-infected insect cells. Biochem Biophys Res Commun 175:285–290
25. Yu VC, Delsert C, Anderson B, Holloway JM, Devray OV, Naar AM, Kim SY, Boutin J, Glass CK, Rosenfeld MG 1991 RXRβ: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. Cell 67:1251–1266
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 receptors to thyroid hormone response elements. J Biol Chem 265:2500–2504
28. Darling DS, Beebe JS, Burnside J, Winslow ER, Chin WW 1991 3,5,3′-Triiodothyronine (T_3) receptor-auxiliary protein (TRAP) binds DNA and forms heterodimers with the T_3 receptor. Mol Endocrinol 4:1627–1635
29. Delegeane AM, Ferland LH, Mellon PL 1987 Tissue-specific enhancer of the human glycoprotein hormone alpha-subunit gene: dependence on cyclic AMP-inducible elements. Mol Cell Biol 7:3994–4002
30. Jameson JL, Albanese C, Habener JF 1989 Distinct adjacent protein-binding domains in the glycoprotein hormone alpha gene interact independently with a cAMP-responsive enhancer. J Biol Chem 264:16190–16196
31. Fiddes JC, Goodman HM 1981 The gene encoding the common alpha subunit of the four human glycoprotein hormones. J Mol Appl Genet 1:3–18
32. Anderson B, Kennedy GC, Hamernick DL, Bokar JA, Bohinski R, Nilson JH 1990 Amplification of the transcriptional signal mediated by the tandem cAMP response elements of the glycoprotein hormone α-subunit gene occurs through several distinct mechanisms. Mol Endocrinol 4:573–582
33. Chatterjee VK, Nagaya T, Madison LD, Datta S, Rentoumis A, Jameson JL 1991 Thyroid hormone resistance syndrome. Inhibition of normal receptor function by mutant thyroid hormone receptors. J Clin Invest 87:1977–1984
34. Sawadogo M, Roeder RG 1985 Factors involved in spe-

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cific transcription by human RNA polymerase II: analysis by a rapid and quantitative *in vitro* assay. Proc Natl Acad Sci USA 82:4394–4398
35. Brent GA, Dunn MK, Harney JW, Gulick T, Larsen PR, Moore DD 1989 Thyroid hormone aporeceptor represses T_3-inducible promoters and blocks activity of the retinoic acid receptor. New Biol 3:329–336
36. Damm K, Thompson CC, Evans RM 1989 Protein encoded by v-*erb*A functions as a thyroid-hormone receptor antagonist. Nature 339:593–597
37. Chatterjee VKK, Madison LD, Mayo S, Jameson JL 1991 Repression of the human glycoprotein hormone α-subunit gene by glucocorticoids: evidence for receptor interactions with limiting transcriptional activators. Mol Endocrinol 5:100–110
38. Strait KA, Schwartz HL, Castillo AP, Oppenheimer JH 1990 Relationship of c-*erb*A mRNA content to tissue triiodothyronine nuclear binding capacity and function in developing and adult rats. J Biol Chem 265:10514–10521
39. Forman BM, Yang C-R, Stanley F, Casanova J, Samuels HH 1988 c-*erb*A protooncogenes mediate thyroid hormone-dependent and -independent regulation of the rat growth hormone and prolactin genes. Mol Endocrinol 2:902–911
40. Privalsky ML, Sharif M, Yamamoto KR 1990 The viral erbA oncogene protein, a constitutive repressor in animal cells, is a hormone-regulated activator in yeast. Cell 63:1277–1286
41. Denner LA, Weigel NL, Maxwell BL, Schrader WT, O'Malley BW 1990 Regulation of progesterone receptor-mediated transcription by phosphorylation. Science 250:1740–1743
42. Bagchi MK, Tsai S-Y, Tsai M-J, O'Malley BW 1990 Regulation of *in vitro* transcription by progesterone receptor. J Biol Chem 265:5129–5134
43. Shupnik MA, Ridgway EC 1987 Thyroid hormone control of thyrotropin gene expression in rat anterior pituitary cells. Endocrinology 121:619–624
44. Ohkuma Y, Horikoshi M, Roedor RG, Desplan C 1990 Engrailed, a homeodomain protein, can repress *in vitro* transcription by competition with the TATA box-binding protein transcription factor IID. Proc Natl Acad Sci USA 87:2289–2293
45. Kato H, Horikoshi M, Roeder RG 1991 Repression of HIV-1 transcription by a cellular protein. Science 251:1476–1478
46. Inoue A, Yamakawa J, Yukioka M, Morisawa S 1983 Filter-binding assay procedure for thyroid hormone receptors. Anal Biochem 134:176–183
47. Macchia E, Nakai A, Janiga A, Sakurai A, Fisfalen ME, Gardner P, Soltani K, DeGroot LJ 1990 Characterization of site-specific polyclonal antibodies to c-*erb*A peptides recognizing human thyroid hormone receptors alpha 1, alpha 2, and beta and native 3,5,3′-triiodothyronine receptor, and study of tissue distribution of the antigen. Endocrinology 126:3232–3239
48. Manley JL, Fire A, Cano A, Sharp PA, Gefter ML 1980 DNA-dependent transcription of adenovirus genes in a soluble whole-cell extract. Proc Natl Acad Sci USA 77:3855–3859

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