In Vitro Transcriptional Studies of the Roles of the Thyroid Hormone (T₃) Response Elements and Minimal Promoters in T₃-stimulated Gene Transcription*

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Thyroid hormone receptors (TRs) are ligand-dependent nuclear transcription factors that are encoded by two different genes, TRα and TRβ, and bind to thyroid hormone response elements (TREs) in the promoters of thyroid hormone (T₃)-regulated genes. Major members of the thyroid hormone receptor auxiliary proteins, have recently been shown to enhance the binding of TRs to TREs. We previously showed that TRs extracted from rat pituitary GH₃ cells retain ligand (T₃) and DNA binding specificity and stimulate rat growth hormone (rGH) promoter activity in a cell-free in vitro transcription system. In this report, we have studied further how T₃ activates endogenous TRs and stimulates transcription from different TRE-containing promoters. We found that T₃ (10⁻⁸ M) selectively stimulates transcription from rGH-TRE-, rGH-TREpal-, and TREpal-, but not ME-TRE- and F₂-TRE-, containing templates in which these TREs are linked in front of the rGH minimal promoter containing only the TATA box binding protein, but not any other proximal binding protein, sequence. In contrast, only the TREpal/AdML template, in which TREpal oligonucleotide was linked in front of the adenovirus major late gene (AdML) minimal promoter, was stimulated by T₃. Electrophoretic mobility shift assay (EMSA) demonstrates that endogenous TR complexes specifically bind to either natural or idealized TRE (rGH-TRE, rGH-TREpal, ME-TRE, and F₂-TRE) oligonucleotides. To further understand these receptor-DNA complexes formed on various TREs, isoform-specific anti-receptor antisera (TRα, TRβ₁, TRβ₂, and RXRβ) were added in the EMSA. These antisera differentially supershifted TR-DNA complexes formed on the TREs. These data suggest that endogenous TR isoforms and RXRβ may form different complexes on the various TREs or that TR-RXR complexes have distinct conformations when bound to the various TREs. Taken together, these data suggest that particular TREs in which specific TR-RXR complexes are formed and different minimal promoters may provide specificity in T₃-mediated transcriptional stimulation of gene expression.

TRs² are encoded by two different genes that are designated TRα and TRβ (1). They are members of the nuclear hormone receptor superfamily and thus contain DNA-binding and ligand-binding domains (2). T₃ regulates gene expression by interaction with TRs that bind to thyroid hormone response elements (TREs) in the promoter of target genes. TREs have been characterized from a number of target genes such as rat growth hormone (rGH) (3), rat α-myosin heavy chain (4), rat malic enzyme (5), chicken lysozyme (6), and rat/mouse/human α and TSHβ subunits (7–10).

TREs generally are composed of dual consensus half-sites, each represented by the sequence: AGGT(C/A)A. The arrangement of the TRE half-sites among different TR-regulated genes is variable (Fig. 1): 1) palindrome, the two TRE half-sites are arranged head-to-head; 2) direct repeat, the two half-sites are oriented head-to-tail, and separated by a gap of any number of nucleotides; and 3) inverted repeat, the half-sites are arranged tail-to-tail and separated by 6 intervening base pairs. Recently, TRs have been shown to heterodimerize with nuclear proteins. Murray and Towle (11) and Burnside et al. (12) first reported that the binding of TRs to TREs is enhanced by nuclear proteins, T₃ receptor auxiliary proteins (TRAPs), from the liver and GH3 cells. Subsequently, several groups have shown that retinoid X receptors (RXRα and RXRβ) are TRAPs, as they enhance the binding to DNA. Moreover, RXRs also augment TR-mediated transcriptional activation (13–17).

Hormone-regulated gene expression, including T₃-mediated transcriptional activation, has been studied by using co-transfection assays in which reporter plasmids containing hormone response elements in front of heterologous promoters and receptor expression plasmids are introduced into different cells (18). However, there are several drawbacks with this approach, such as variable efficiency of transfection and the unphysiological overexpression of receptors in the cell. In addition, little is known about how T₃ activates endogenous TRs and stimulates the transcription of its target genes. We have recently developed a ligand (T₃) and TR-dependent cell-free in vitro transcription system and found that T₃-mediated transcriptional stimulation of the rGH gene expression is independent of the Pit-1/GHF-1 binding sites (19). To understand better how T₃ activates TRs that bind to distinct TREs in the promoter region of different target genes and stimulates gene transcription, we have used our cell-free in vitro transcription system to examine T₃-stimulated transcription from several different TRE-containing promoters. These promoters contain natural and/or idealized TRE oligonucleotides that are ligated in front of either the rat growth hormone gene

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²The abbreviations used are: TR, thyroid hormone receptor; TRE, thyroid hormone response element; T₃, thyroid hormone; rGH, rat growth hormone; TSH, thyroid-stimulating hormone, TRAP, thyroid hormone receptor auxiliary protein; AdML, adenovirus major late gene; EMSA, electrophoretic mobility shift assay; bp, base pairs; DTT, dithiothreitol; CAT, chloramphenicol acetyltransferase; RAR, retinoic acid receptor; RXR, retinoic acid X receptor; RARE, retinoic acid response element; VDR, vitamin D receptor; VDRE, vitamin D response element.
with an ice-cold glass homogenizer to prepare the nuclei. After 30 strokes, the preparation was centrifuged (15,000 × g, 10 min, 2 °C), and the supernatant was removed. The nuclei were resuspended in 1 nuclear pellet volume of extraction buffer (20 mM Hepes, pH 7.9, 25% glycerol, 0.5 mM KCl, 3 mM MgCl₂, 0.75 mM spermidine, 0.15 mM spermine, 0.5 mM EDTA, 2 mM DTT, and protease inhibitor mixture). After extraction for 40 min, the preparation was centrifuged (20,000 × g, 20 min, 2 °C), and the supernatant was collected and dialyzed against nuclear dialysis buffer (20 mM Hepes, pH 7.9, 20% glycerol, 60 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT, and 1 mM phenylmethylsulfonyl fluoride) for 4 h.

Electrophoretic Mobility Shift Assay (EMSA)—Oligonucleotides were end-labeled with T4 polynucleotide kinase in the presence of [γ-³²P]ATP. GH₃ nuclear extract (4–5 mg/ml) was incubated with the gel-purified ³²P-labeled probe (0.5 ng) on ice for 30 min in the presence of the EMSA buffer (20 mM Hepes, pH 7.9, 60 mM KCl, 2 mM DTT, 5% glycerol, 1 mg/ml bovine serum albumin, 5 mM MgCl₂, 2 mM phenylmethylsulfonyl fluoride, 4 μg of poly(dI/dC), and 2 μg of sonicated salmon sperm DNA). For the antibody-mediated supershift experiments, ³²P-labeled probes were incubated with the GH₃ nuclear extract for 30 min. Different amounts of the isoform-specific receptor antibodies were then added, and reactions were kept at 4 °C for another 2 h. Protein/DNA complexes were separated from the free DNA probe by electrophoresis through a 5% polyacrylamide gel in 0.5 × Tris-borate-EDTA buffer for 90 min at 4 °C; the gel was dried and then subjected to autoradiography.

Cell-free In Vitro Transcription Assay—A typical cell-free in vitro transcription reaction has been described previously (19). In brief, a transcription reaction mixture containing 20 mM Hepes, pH 7.9, 60 mM KCl, 6 mM MgCl₂, 2 mM DTT, 0.1 mM EDTA, 15% glycerol, 0.5 mM ATP, 0.5 mM CTP, 20 mM unlabeled UTP, 25 μCi of [α-³²P]UTP, 1 mM 3'-O-methyl GTP, 5 mM creatine phosphate, 20 units of RNase T1, 0.5 μg of sonicated salmon sperm DNA, 15 μg of GH₃ nuclear extract, 100 ng of the test template, 100 ng of internal control template (AdML/G), and 10⁻⁸ M T₃ was incubated at 30 °C for 90 min. The reactions were terminated by adding 70 μl of the stop mixture (25 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% SDS, 200 μg/ml yeast tRNA, 400 μg/ml proteinase K) and incubated for 30 min at 37 °C. After two extractions with phenol-chloroform-isooamyl alcohol (24:24:1), RNA was precipitated and subjected to electrophoresis through a 6% polyacrylamide-7 M urea sequence gel. Autoradiography of dried gels was performed at ~80 °C with an intensifying screen.

RESULTS

Selective Activation of Different TREs by T₃-activated TRs in Vitro—T₃ regulates gene expression by binding to TRs that interact with different TREs in the promoter region of the target genes (1). However, little is known about the mechanism by which this regulation occurs. In this study, we examined T₃-stimulated transcription from different TRE-containing templates, in which single copies of the rGH-TRE, TREpal, F₂-TRE, and ME-TRE were ligated in front of the rGH gene (Fig. 2A) minimal promoter, in a cell-free transcription system. As shown in Fig. 2B, the transcriptional stimulation from the rGH-TRE/rGH template was increased 4-fold by T₃ (10⁻⁸ M) (Fig. 2B, compare lanes 1 and 2) which is in agreement with our previous data (19). T₃ also caused a 4-fold transcriptional stimulation from the TREpal/rGH template (Fig. 2B, compare lanes 3 and 4). These results also are consistent with previous results using the transient transfection assay (3, 23, 24). However, neither the F₂-TRE/rGH (Fig. 2B, lanes 5 and 6) nor the ME-TRE/rGH (Fig. 2B, lanes 7 and 8) templates showed any significant activity.

We then tested the specificity of the transcriptional stimulation from the rGH-TRE/rGH or TREpal/rGH templates. Twenty-fold molar excess of the rGH-TRE (Fig. 2C, lane 4) or TREpal (Fig. 2C, lane 7) competed with the GH₃ nuclear extract for 10 min, and the transcription reactions were performed at 30 °C for another 60 min. T₃-mediated transcriptional stimulation from the rGH-TRE/rGH (Fig. 2C, compare lanes 1, 3, and 4) and TREpal/rGH (Fig. 2C, compare lanes 2, 5, and 6) templates was blocked. Transcription from the AdML/G template, an internal control, did not change in the presence of T₃ (10⁻⁸ M). We also showed that

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**Experimental Procedures**

Oligonucleotides and Plasmids—The sequences of the natural and idealized TRE oligonucleotides used in the EMSA are shown in the Fig. 1. All of the templates used in the in vitro transcription assays were constructed by standard methods (20). rGH-TATA/G plasmid was constructed by insertion of a rGH minimal promoter oligonucleotide (~50 to ~1) into the BglII/SacI sites of the pLovTATA plasmid (21) in a which a 380-bp guanosine-less cassette (G-less cassette) is located adjacent to the SacI site. rGH-TRE/rGH, TREpal/GF₂, F₂-TRE/AdML, and ME-TRE/AdML plasmids were prepared by ligating these TRE oligonucleotides, with HindIII/BamHI sites at each end, into the pML/C2AT (19) -51 plasmid (22), which contains a 380-bp guanosine-less cassette under the control of the adenovirus major late protein gene minimal promoter. AdML/G plasmid that contains the AdML promoter (~400 to ~1) linked to a short form G-less cassette (200 bp) was added as an internal control in all of the in vitro transcription assays.

**Cell Culture and Preparation of Nuclear Extract—or Crude nuclear transcription extract from the GH₃ cells was prepared as described previously (19). Briefly, cells were harvested by centrifugation and washed in ice-cold phosphate-buffered saline. The cell pellets were resuspended in the hypotonic buffer (10 mM Hepes, pH 7.9, 1 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 2 mM dithiothreitol (DTT), and 2 mM phenylmethylsulfonyl fluoride, and swollen in ice for 10 min. The cell suspension was spun down and resuspended in 2 original cell volumes of hypotonic buffer plus protease inhibitor mixture (2 mM phenylmethylsulfonyl fluoride, 10 mg of leupeptin per ml, 5 mg of aprotinin per ml). The cells were then disrupted

**FIG. 1. Thyroid hormone response elements.** The TREs used in this study are shown. Native TREs—rGH-TRE (rat growth hormone gene promoter, ~200 to ~160), ME-TRE (malic enzyme gene promoter, ~288 to ~254), F₂-TRE (chicken lysozyme gene promoter, ~2354 to ~2328). Synthetic TRE: TREpal (palindromic thyroid hormone response element). The location and orientation of TRE half-sites are indicated by arrows.

(rGH) and/or adenovirus major late gene (AdML) minimal promoters. We found that T₃ selectively stimulates transcription from these TRE-containing templates. However, the electrophoretic mobility shift assay (EMSA) showed that endogenous TRs can form specific receptor-DNA complexes on these TREs. Nevertheless, isoform-specific anti-receptor antisera (TRα, TRβ1, TRβ2, and RXRβ) can differentially supershift these receptor-DNA complexes. These results suggest that TRs and RXRs in the GH₃ nuclear extract may form distinct complexes on these TREs. In sum, these data suggest that T₃-stimulated transcription from the different TRE-containing templates may depend upon both 1) the particular TREs that enable the formation of TR-RXR complexes with either different composition and/or conformation and 2) the context of minimal promoter of different genes.
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A

rGH-TRE/rGH:

- - - +

rGH-TATA

G-free 380

TREpal/rGH:

- - - +

rGH-TATA

G-free 380

Fz-TRE/rGH:

- - - +

rGH-TATA

G-free 380

ME-TRE/rGH:

- - - +

rGH-TATA

G-free 380

B

T3:

- - - - - +

reporter

internal control

1 2 3 4 5 6 7 8

FIG. 2. *In vitro* analysis of T3-stimulated transcription from TRE-containing templates in the context of the rGH minimal promoter. A, schematic diagram of the reporter templates used in the *in vitro* transcription assay. A single copy of different TRE (rGH-TRE, TREpal, Fz-TRE, and ME-TRE) oligonucleotides was ligated in front of the rGH minimal promoter (rGH TATA) that is immediately adjacent to the 380-bp G-less cassette. B, selective transcriptional stimulation from the TRE-containing templates by T3. GH3 nuclear extract (4-5 mg/ml) was incubated with (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) 10^{-6} M T3 for 10 min. 100 ng of the rGH-TRE/rGH (lanes 1 and 2), TREpal/rGH (lanes 3 and 4), Fz-TRE/rGH (lanes 5 and 6), ME-TRE/rGH (lanes 7 and 8) templates and the same amount of the internal control template were added, and reactions were kept at 30°C for another 60 min. After phenol/chloroform extraction and ethanol precipitation, RNA transcripts were subjected to electrophoresis on 6% polyacrylamide-7 M urea sequencing gels. C, specificity of T3-stimulated transcription from the rGH-TRE/rGH and TREpal/rGH templates. Transcription reactions from the rGH-TRE/rGH (lanes 1, 3, and 4) and TREpal/rGH (lanes 2, 5, and 6) templates were the same as described in B. T3 (10^{-6} M) (lanes 3, 4, 5, and 6) was added to the GH3 nuclear extract. For the competition study, a 20-fold molar excess of either the rGH-TRE (lane 4) or TREpal (lane 6) oligonucleotides was added with the GH3 nuclear extract, prior to the addition of T3 (10^{-6} M) and reporter templates. D, basal transcription from the either the rGH-TATA/G or rGH-TRE/rGH templates. Transcription reactions from the rGH-TATA/G (a rGH minimal promoter) (lane 1) and rGH-TRE/rGH (lanes 2-5) templates were the same as described in B. For the competition study, a 30-fold molar excess of the rGH-TRE oligonucleotide was incubated with GH3 nuclear extract either in the absence (lane 3) or in the presence (lane 5) of T3 (10^{-6} M).

addition of the rGH-TRE in front of the rGH minimal promoter did not affect the basal transcription from the rGH minimal promoter (Fig. 2D; compare lanes 1 and 2). In addition, preincubation of GH3 nuclear extract with 30-fold molar excess of rGH-TRE oligonucleotide did not affect the basal transcription from the rGH-TRE/rGH template (Fig. 2D; compare lanes 2 and 3).

To investigate further the nature of the selective T3-induced transcription from different TRE-containing promoters, we next examined whether the specific minimal promoter may play a role. The minimal promoter of the AdML gene is one of the strongest promoters studied. It has been well characterized and does not contain a TRE half-site (22). rGH-TRE, TREpal, Fz-TRE, and ME-TRE oligonucleotides were ligated in front of the AdML gene minimal promoter (Fig. 3A), and the standard *in vitro* transcription reactions were performed in the presence of T3 (10^{-6} M). T3 stimulated transcription by 6- to 7-fold from the TREpal/AdML template (Fig. 3B, compare lanes 3 and 4). However, T3 did not stimulate transcription from the rGH-TRE/AdML template (Fig. 3B, compare lanes 1 and 2). The inability of T3 to stimulate transcription from the rGH-TRE/AdML template is similar to the transient transfection assay results, as T3 did not stimulate chloramphenicol acetyltransferase (CAT) activity from the reporter gene in which the rGH-
TRE was cloned upstream of the Rous sarcoma virus (RSV) minimal promoter (24). As before, neither the ME-TRE/AdML template (Fig. 3B, compare lanes 5 and 6) nor the F2-TRE/AdML (Fig. 3B, compare lanes 7 and 8) templates showed any T3-mediated transcriptional stimulation.

To verify the specificity of the T3-stimulated transcription from the TREpal/AdML template, GH3 nuclear extract was preincubated with 20-fold molar excess of the TREpal oligonucleotide (Fig. 3C, lane 3). T3-stimulated transcriptional activity (Fig. 3C, lane 2) was decreased to basal levels (Fig. 3C, compare lanes 1 and 3). However, a 20-fold molar excess of the Pit-1/GHF-1 oligonucleotide, containing the sequences from -135 to -60 of the RGH promoter, did not affect T3-mediated transcriptional stimulation from the TREpal/AdML template (Fig. 3C, compare lanes 2 and 4). In transfection assays, it has been shown that T3 stimulates chloramphenicol acetyltransferase (CAT) activity from either F2-TRE (6) or ME-TRE (5) containing templates in which these TREs are ligated in front of the thymidine kinase promoter. However, we observed that T3 did not stimulate transcription from the F2-TRE- and ME-TRE-containing templates in either the RGH gene or AdML gene minimal promoter of our in vitro transcription system. To confirm the former observation, we constructed a template in which a single copy of the F2-TRE oligonucleotide was ligated in front of the thymidine kinase promoter. We found that T3 (10^-8 M) indeed stimulates transcription in vitro from this template (Fig. 4, compare lanes 1 and 2) which is consistent with that from the transient transfection assay (6). This transcriptional stimulation can be inhibited by preincubation of a 20-fold molar excess of F2-TRE oligonucleotide with the GH3 nuclear extract (Fig. 4, compare lanes 2 and 3). These results indicate that T3 selectively stimulates transcription from different TRE-containing promoters, and the minimal promoter of different genes can greatly influence this T3-mediated transcriptional activity.

Formation of Specific Receptor-DNA Complexes on Different TREs—The selective inability of several TRE-containing templates to mediate T3-regulated transcription led us to investigate further whether endogenous TRs can bind to these TREs. [33P]Labeled oligonucleotides containing various arrangements of TRE half-sites (Fig. 1) were incubated with GH3 nuclear extract, and EMSA was performed. Fig. 5A shows that two specific receptor-DNA complexes are formed by adding increasing amounts of GH3 nuclear extract to either the rGH-TRE (Fig. 5A, lanes 1-3) or F2-TRE (Fig. 5A, lanes 4-6) oligonucleotide probes. However, F2M, a mutated F2-TRE in which a half-site G-chloramphenicol acetyltransferase (CAT) activity from either the RGH gene or AdML gene minimal promoter of our in vitro transcription system. To confirm the former observation, we constructed a template in which a single copy of the F2-TRE oligonucleotide was ligated in front of the thymidine kinase promoter. We found that T3 (10^-8 M) indeed stimulates transcription in vitro from this template (Fig. 4, compare lanes 1 and 2) which is consistent with that from the transient transfection assay (6). This transcriptional stimulation can be inhibited by preincubation of a 20-fold molar excess of F2-TRE oligonucleotide with the GH3 nuclear extract (Fig. 4, compare lanes 2 and 3). These results indicate that T3 selectively stimulates transcription from different TRE-containing promoters, and the minimal promoter of different genes can greatly influence this T3-mediated transcriptional activity.
Thymidine kinase minimal promoter that is immediately adjacent to a receptor-DNA complex was blocked by preincubation of the RXRp (lanes 7-12) oligonucleotides as increasing amounts of the GH3 nuclear extract was added in the EMSA. The formation of the receptor-DNA complex was blocked by preincubation of the GH3 nuclear extract with 10-fold (Fig. 5B, lanes 3 and 9) and 25-fold (Fig. 5B, lanes 4 and 10) molar excess of either unlabeled ME-TRE or TREpal oligonucleotides, respectively. Additionally, there was no specific receptor-DNA complex formed in the absence (lane 1) or presence (lanes 2 and 3) of T3 (10^{-8} M). For the competition study, a 30-fold molar excess of F2-TRE oligonucleotide (lanes 1-6) was preincubated with GH3 nuclear extract.

Receptor-DNA Complexes Formed on Various TREs Differentially Supershifted by Isoform-specific Anti-TRs and Anti-RXRβ Antibodies—To further characterize these receptor-DNA complexes formed on different TREs, isofrom-specific anti-TR (TRα, TRβ1, and TRβ2), anti-retinoid X receptor (RXRβ), and anti-retinoic acid receptor (RAR) antisera, whose specificity has been confirmed (15, 26), were added to the EMSA. Anti-RXRβ (Fig. 6A, lane 2) and anti-RXRβ (lane 3) antisera decreased receptor-DNA complexes formed on the 32P-labeled TREpal oligonucleotide and supershifted them to slower mobility complexes (indicated by arrowheads). As previously shown, two receptor-DNA complexes were formed on the 32P-labeled rGH-TRE oligonucleotide (19). Anti-RXRβ antisera decreased the formation of the faster mobility receptor-DNA complex (Fig. 6B, lane 2) without changing that of the slower mobility receptor-DNA complex. Anti-RXRβ antisera reduced the amount of both receptor-DNA complexes (Fig. 6B, lane 3) while both antisera supershifted these receptor-DNA complexes to ones with slower and different mobilities (Fig. 6B, indicated by arrowheads). Anti-TRα, anti-TRβ1, and anti-RAR antisera were also added to the EMSA. They neither decreased formation of receptor-DNA complexes nor caused the antisera-mediated receptor-DNA supershift on either the TREpal or rGH-TRE oligonucleotides (Table I).

In order to rule out the possibility that differential avidities among these antibodies contributed to these results, we also performed an experiment in which increasing amounts of antisera were added to the EMSA. Fig. 6C shows that the addition of increasing amounts of anti-TRβ2 antisera (Fig. 6C, lanes 7-12) oligonucleotides as well as anti-TRα antisera (lanes 1-6) also supershifted the faster mobility receptor-DNA complex (Fig. 6C, lane 3). These findings suggest that the differential avidities among these antibodies do not contribute to these results.

![Fig. 4. T3 stimulates transcription from the F2-TRE-containing template. A single copy of the F2-TRE was ligated in front of the thymidine kinase minimal promoter that is immediately adjacent to a 380-bp G-less cassette. Standard in vitro transcriptions were performed in the absence (lane 1) or presence (lanes 2 and 3) of T3 (10^{-8} M). For the competition study, a 30-fold molar excess of F2-TRE oligonucleotide (lane 3) was preincubated with GH3 nuclear extract.](image)

![Fig. 5. Binding of endogenous receptors to different TREs by EMSA. A, 32P-labeled rGH-TRE (lanes 1-3), F2-TRE (lanes 4-6), and F2M, a mutated F2-TRE containing only one TRE half-site (lane 7) oligonucleotides were incubated with 0.5 ml (lanes 1 and 4), 1 ml (lanes 2 and 5), and 2 ml (lanes 3, 6, and 7) of GH3 nuclear extract (4-5 mg/ml). B, 32P-labeled ME-TRE (lanes 1-6) and TREpal (lanes 7-12) oligonucleotides were incubated with either 0.5 ml (lanes 1, 5, 7, and 11) or 2 ml (lanes 2, 3, 4, 6, 8, 9, 10, and 12) of GH3 (lanes 1-6) and HeLa (7-8 mg/ml) (lanes 7-12) nuclear extracts for 30 min. For the competition experiment, 10-fold (lanes 3 and 9) and 25-fold (lanes 4 and 10) molar excess of unlabeled ME-TRE (lanes 3 and 4) and TREpal (lanes 9 and 10) oligonucleotides were preincubated with the GH3 nuclear extract. Receptor-DNA complexes were separated through a 5% polyacrylamide gel, and specific receptor-DNA complexes are indicated by arrows.](image)
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Fig. 6. Supershift receptor-DNA complexes are differentially formed on various TREs by isoform-specific anti-TRα, anti-TRβ, and anti-RAR antibodies. 32P-Labeled TRE probes were incubated with 1 μl of GH3 nuclear extract (4–5 mg/ml) for 30 min, and isoform-specific anti-TRα, anti-TRβ, and anti-RAR antibodies were added to the reaction mixture for another 90 min. The receptor-DNA complexes were separated through a 5% nondenatured polyacrylamide gel and are indicated by arrowheads. A, 32P-labeled TREpal oligonucleotide. Lane 1, no antiserum added; lane 2, TRβ2-specific antiserum (1 μl); and lane 3, RXRβ-specific antiserum (1 μl). B, 32P-labeled rGH-TRE oligonucleotide. Lane 1, no antiserum added; lane 2, TRβ2-specific antiserum (1 μl); and lane 3, RXRβ-specific antiserum (1 μl). C, 32P-labeled TREpal oligonucleotide. Lane 1, no antiserum added; lanes 2–4, 0.5, 1, and 3 μl of the TRβ2-specific antiserum; lanes 5–7, 0.5, 1, and 3 μl of the TRα antiserum; lanes 8–10, 0.5, 1, and 3 μl of the TRα1-specific antiserum; and lanes 11 and 12, 1 and 3 μl of the TRα2-specific antiserum alone, without GH3 nuclear extract. D, 32P-labeled F2-TRE oligonucleotide. Lane 1, no antiserum added; lane 2, TRβ2-specific antiserum (1 μl); lane 3, TRα2-specific antiserum (1 μl); lane 4, TRα1-specific antiserum (1 μl); and lane 5, TRβ1-specific antiserum (1 μl). E, 32P-labeled ME-TRE oligonucleotide. Lane 1, no antiserum added; lane 2, TRβ1-specific antiserum (1 μl); lane 3, TRα2-specific antiserum (1 μl); lane 4, TRβ2-specific antiserum (1 μl); and lane 5, RVRβ-specific antiserum (1 μl).
incubation of these isoform-specific anti-receptor antisera with but not anti-TRp2 (lane 1) resulted in progressively decreasing the formation of the receptor-DNA complex in Ts-stimulated gene expression. In vitro translated TR.RAR, but not TR.RXR, heterodimer binding to either the rME-TRE or the MBP-TRE (33). Our data also indicate that T₃ selectively stimulates transcription from the rGH-TRE/rGH and TREpal/rGH, but not F₂₃-TRE/rGH and ME-TRE/rGH templates. The inability of transcriptional stimulation from the F₂₃-TRE- and ME-TRE-containing templates by T₃ was not due to the inability of the binding of endogenous TRs to these TREs. In combination with the previous results, our findings also indicate that effective receptor-DNA binding as determined by EMSA does not necessarily correlate with transcriptional activation from the same TRE and suggest that specific receptor-DNA complexes and specific minimal promoter may be critical for T₃-stimulated transcription in vivo and in vitro.

We have previously shown that nuclear extract from GH₃ cells contains authentic TRs (19). Recently, Davis and Lazar (34) also reported the existence of RXRs and RXRβ mRNA in the GH₃ cells. To understand the nature of the interaction among endogenous TRs and RXRs and the possible TR-RXR complexes that bind to various TREs, we have demonstrated that endogenous TRs and RXRβ in the GH₃ nuclear extract can form isoform-specific and different receptor-DNA complexes on natural and idealized TRE-containing oligonucleotides. Therefore, it is possible that different TREs have distinct antisera-mediated supershift patterns, and this is not due to the different avidities among these antisera. These results suggest that different TR isoforms and RXRβ may form distinct complexes on various TREs or TR-RXR complexes formed on various TREs may have different conformations that can be recognized by isoform-specific anti-TR and anti-RXR antisera. Carberg et al. (35) recently demonstrated the existence of two functionally distinct classes of vitamin D response elements (VDREs) in the mouse osteopontin and human osteocalcin genes. The VDRE from the human osteocalcin gene, which binds to vitamin D receptor (VDR) as a homodimer, confers vitamin D inducibility mediated by VDR alone. In contrast, the VDRE from the mouse osteopontin gene is synergistically stimulated by vitamin D and 9-cis-retinoic acid-activated VDR-RXR heterodimers. Stunnenberg (36) also suggests that RAR-RXR heterodimers with different conformations might exist within the cell having the ability to transactivate either through a RARE, direct repeat half-site with 5-nucleotide spacing (DR+5), as in the RA receptor β2 (RARβ2) promoter or RARE, direct repeat half-site with 1-nucleotide spacing (DR+1), as found in the promoter of the rat cellular retinoic acid binding protein type I. Therefore, it is possible that distinct TREs in the promoter

### Table 1

|            | rGH-TRE | ME-TRE | TREpal | F₂₃-TRE |
|------------|---------|--------|--------|---------|
| TR antibody (α) | -       | -      | -      | +       |
| TR antibody (β₁) | -       | +      | +      | -       |
| TR antibody (β₂) | +       | +      | +      | -       |
| RXR antibody (β) | +       | -      | +      | -       |
| RAR antibody    | -       | -      | -      | -       |

DISCUSSION

Bacterially expressed (27, 28) and in vitro translated (29) TRs bind to various TREs as monomers, homodimers, and as heterodimers in association with TRAPs. RXRs, members of steroid/thyroid hormone receptor superfamily, have recently been shown to behave similarly to TRAPs by heterodimerizing with TRs, enhancing TR binding to its cognate TREs, and augmenting T₃-mediated transcriptional activation (13-17). Results from co-immunoprecipitation and cross-linking experiments also indicate that TRs and RXRs are favored to form heterodimers in solution, and only the heterodimers bind effectively to the TREs (13, 16, 17). Several laboratories also reported that T₃ inhibits either the in vitro translated (30) or bacterial expressed (27) TR homodimer formation on TREs. In addition, Yen et al. (25) also reported recently that T₃ decreases in vitro translated TR-RAR, but not TR-RXR, heterodimer binding to the TREs. Taken together, these data suggested that the TR-RXR heterodimer may be a physiologically important receptor complex in T₃-stimulated gene expression. In a recent study, Nagpal et al. (31) reported that productive functional homodimeric RAR interactions do not occur in transfected cells under conditions where heterodimeric RAR-RXR interaction can be readily detected. Their results also suggest that RAR-RXR heterodimerization is preferred in vitro over RAR homodimerization.

TREs, naturally present in different T₃-regulated target genes, exhibit an unexpected diversity (with differences in either spacing and/or orientation of the TRE hexamer half-sites) (Fig. 1). However, when in vitro translated TRα and TRβ are mixed with RXRα and RXRβ in the EMSA, the formation of TR-RXR heterodimers, with enhanced DNA binding, is observed on either the natural or idealized TREs (13-17). An important question is whether the formation of heterodimers and the synergism observed in binding between the TRs and RXRs to different TREs can be directly correlated with the transcriptional activation of the heterodimers on their response elements. Hermann et al. (32) first reported that a strong synergistic transactivation from the rMHC-TRE (rat α-myosin heavy chain-TRE), but not rME-TRE, was observed when TRα and RXRα were co-transfected into the CV-1 cells although effective TRα-RXRα heterodimer binding to the rMHC-TRE and rME-TRE were observed in EMSA. Hallenbeck et al. (33) also reported that TRα-RXRβ heterodimer can be activated by T₃ only from the rME-TRE, but not from the MBP-TRE (myeloid basic protein-TRE), containing promoter in a transient transfection assay. Remarkably, EMSA revealed no difference in the TRα-RXRβ heterodimer binding to either the rME-TRE or the MBP-TRE (33). Our data also indicate that T₃ selectively stimulates transcription from the rGH-TRE/rGH and TREpal/rGH, but not F₂₃-TRE/rGH and ME-TRE/rGH templates. The inability of transcriptional stimulation from the F₂₃-TRE- and ME-TRE-containing templates by T₃ was not due to the inability of the binding of endogenous TRs to these TREs. In combination with the previous results, our findings also indicate that effective receptor-DNA binding as determined by EMSA does not necessarily correlate with transcriptional activation from the same TRE and suggest that specific receptor-DNA complexes and specific minimal promoter may be critical for T₃-stimulated transcription in vivo and in vitro.

We have previously shown that nuclear extract from GH₃ cells contains authentic TRs (19). Recently, Davis and Lazar (34) also reported the existence of RXRs and RXRβ mRNA in the GH₃ cells. To understand the nature of the interaction among endogenous TRs and RXRs and the possible TR-RXR complexes that bind to various TREs, we have demonstrated that endogenous TRs and RXRβ in the GH₃ nuclear extract can form isoform-specific and different receptor-DNA complexes on natural and idealized TRE-containing oligonucleotides (Fig. 1). Using isoform-specific antisera to the TRs (α, β₁, and β₂), RXRβ, and RAR in EMSA, we show that they can differentiate supershift endogenous TR-RXR complexes formed on different TREs (Fig. 6; Table I). This is the first demonstration that endogenous receptor-DNA complexes formed on different TREs have distinct antisera-mediated supershift patterns, and this is not due to the different avidities among these antisera. These results suggest that different TR isoforms and RXRβ may form distinct complexes on various TREs or TR-RXR complexes formed on various TREs may have different conformations that can be recognized by isoform-specific anti-TR and anti-RXR antisera. Carberg et al. (35) recently demonstrated the existence of two functionally distinct classes of vitamin D response elements (VDREs) in the mouse osteopontin and human osteocalcin genes. The VDRE from the human osteocalcin gene, which binds to vitamin D receptor (VDR) as a homodimer, confers vitamin D inducibility mediated by VDR alone. In contrast, the VDRE from the mouse osteopontin gene is synergistically stimulated by vitamin D and 9-cis-retinoic acid-activated VDR-RXR heterodimers. Stunnenberg (36) also suggests that RAR-RXR heterodimers with different conformations might exist within the cell having the ability to transactivate either through a RARE, direct repeat half-site with 5-nucleotide spacing (DR+5), as in the RA receptor β2 (RARβ2) promoter or RARE, direct repeat half-site with 1-nucleotide spacing (DR+1), as found in the promoter of the rat cellular retinoic acid binding protein type I. Therefore, it is possible that distinct TREs in the promoter
region of different target genes may bind to particular TR-RXR complexes and have differential roles in regulating the transcription of particular target genes.

There is increasing evidence indicating that the structure of the minimal promoter confers specificity of the response to certain cis-acting elements and transcription factors. For example, a muscle-specific enhancer from the myoglobin promoter does not activate transcription from the proximal element of the SV40 promoter but does activate transcription from its own minimal promoter (37). Similarly, the effects of different upstream activators have been compared with different TATA motifs using the human hsp70 gene promoter. The activator CP1 was found to stimulate transcription regardless of the TATA box. In contrast, the factor ATF was highly stimulatory in conjunction with the hsp70 and adenovirus E1A TATA boxes, but was weakly activating in the presence of the SV40 TATA box (38). Simon et al. (39) also reported that adenovirus E1A protein-stimulated transcription from the e-fos minimal promoter is TATA sequence-specific, since substitution of the c-fos TATA box with a TATA box from the adenovirus E3 early promoter abolishes its E1A inducibility. In this report, we have also shown that transcription from the RGH-TRE that was ligated in front of the RGH, but not AdML, promoter, and from the F2-TRE that was ligated in front of the thymidine kinase, but not RGH and AdML promoter, were selectively stimulated by T3 in a cell-free in vitro transcription system. This selective stimulation of gene expression in response to different hormone response elements from the heterologous promoter was also observed in the transient transfection studies. For example, it has been reported that T3-stimulated CAT activity from the reporter gene in which RGH-TRE was ligated in front of either the RGH or thymidine kinase (23), but not Rous sarcoma virus (24), minimal promoters. Berkenstam et al. (40) also reported that RA strongly induces luciferase activity from the reporter gene in which RARE was ligated in front of RA receptor β2 (RARβ2), but not thymidine kinase, minimal promoter. In addition, Nagpal et al. (41), using co-transfection of different RAR and RXR isoforms with several reporter plasmids containing either naturally occurring RA-responsive promoter regions or identified RA response elements (RAREs) in COS-1 cells, showed that RA mediated transcriptional activation exhibits specific patterns that are dependent upon both the promoter context and specific RAREs. Finally, we have also found that the minimal promoter of RGH and AdML genes can form different and specific nuclear protein-DNA complexes with nuclear extracts from the GH3 cells (data not shown). Taken together, these results imply that the minimal promoter of different genes may bind to different sets of basal transcription factors that can interact with various upstream activator proteins in the regulation of expression of different genes.

In summary, our results indicate that 1) T3 selectively stimulates transcription from different TRE-containing templates; 2) endogenous receptors (TRs and RXRs) present in the GH3 nuclear extract bind specifically to either natural or idealized TRES; and 3) isoform-specific TRs and RXRs are differently interspersed receptor-DNA complexes formed on various TRES. Taken together, these results suggest T3-stimulated transcription from different target genes may be dependent upon the interaction between the specific TR-RXR complexes formed on these different TRES and the specific nuclear protein-DNA complexes formed on different minimal promoters. It is not yet known whether the TRs and/or RXRs can interact directly with the proteins bound to the minimal promoter or require additional proteins (adaptors) to activate gene expression. We are currently investigating mechanisms by which the endogenous TR and RXR isoforms formed on various TRES interact directly and/or indirectly with general transcriptional apparatus. Recently, Mader et al. (42) have reported that multiple factors are involved in the transcriptional activation from the chimeric promoters containing various RARE or RXRE oligonucleotides by RARs and RXRs. Our findings also suggest that TR-RXR complexes formed on various TRES may provide unique surfaces for interaction with either proximal binding proteins and/or general transcription initiation complexes in the regulation of expression of target genes.

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