Thyroid hormone receptors bind to thyroid hormone response elements (TREs) as heterodimers with T3,5,3'-triiodothyronine (T3) receptor auxiliary protein (TRAP) and retinoid X receptors (RXRs). Currently, it is not known whether TR/TRAP or TR/RXR heterodimers need to bind to both TRE half-sites and whether there is a preferred orientation for TR/RXR heterodimer binding to TREs or transcriptional activation. Accordingly, we created a mutant TRα (TR-P box) by changing 3 amino acids in the P box region of the first zinc finger of the DNA-binding domain to that of the glucocorticoid receptor (GR), and we examined wild-type TRα and TR-P box complex binding to hybrid response elements containing TRE and glucocorticoid receptor element (GRE) half-sites arranged as a direct repeat with a four-nucleotide gap. TR-P box/RXR heterodimers selectively bound to the hybrid response element in which the GRE half-site was the downstream half-site, whereas TRα/RXR bound to hybrid response elements in which GREs were in either position. Additionally, TR/ TRAP or TR/RXR heterodimer required two half-sites for binding to DNA, with strong binding to at least one of the half-sites. Last, co-transfection assays and methylation interference studies using the hybrid response elements suggest that the sequential arrangement of strong and weak half-sites in the TRE may be a critical determinant of TR/RXR heterodimer binding and transcriptional activation.

First, there are two major TR isoforms, TRα and TRβ, which are encoded on separate genes (1, 2). Unlike some members of this superfamily, such as the estrogen and glucocorticoid receptors (1, 2) which bind to conserved hormone response elements (HREs) in the promoter region of target genes, TR binding to thyroid hormone response elements (TREs) appears to be a more complex process.

Second, we and others (7, 18–21) recently showed that T3 itself can affect the nature of the receptor complexes that bind to TREs as T3 decreases TR homodimer, but not TR/ TRAP or TR/RXR heterodimer, binding to some TREs. On the basis of these data, and co-transfection experiments which showed that unliganded TRs repress basal transcription (22, 23), we have proposed that unliganded TR homodimers, when bound to TREs, may repress basal transcription of target genes which is relieved when ligand causes dissociation of TR homodimers from TREs. TR/ TRAP and TR/RXR heterodimers remain bound to TREs in the presence of T3, further supporting their potential roles in mediating transcriptional activation above basal transcription levels.

Half-site Arrangement of Hybrid Glucocorticoid and Thyroid Hormone Response Elements Specifies Thyroid Hormone Receptor Complex Binding to DNA and Transcriptional Activity*

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The abbreviations used are: TR, thyroid hormone receptor; HRE, hormone response element; TRE, thyroid hormone receptor element; T3, T3,5,3’-triiodothyronine; TRAP, TR receptor auxiliary protein; RXR, retinoid X receptor; DR, direct repeat; IP, inverted palindrome; GR, glucocorticoid receptor; GRE, glucocorticoid receptor element; EMSA, electrophoretic mobility shift assay.

1. Current, it is not known whether TR/ TRAP needs to bind to both TRE half-sites to form heterodimers, and whether there is a preferred orientation for TR/ TRAP heterodimer binding to TREs or transcriptional activation. Recently, Zhou et al. (30) developed an “oriented heterodimer” approach to study the orientation of the catalytic subunit of a bacterial transcription activator when bound to DNA. We now have used this approach to study TR/ TRAP and TR/RXR heterodimer binding to TREs and transcriptional activation.

In these studies, we have created a mutant TRα (TR-P box)
by changing 3 amino acids in the P box region of the first zinc finger of the DNA-binding domain to that of the glucocorticoid receptor (GR). This region has previously been shown to be important for TR, GR, and estrogen receptor recognition of their respective HREs (31–34), so TR-P box should have preferential binding to glucocorticoid response element (GRE) half-sites over TRE half-sites. We then compared the DNA binding of the TR-P box with wild-type TRα to hybrid response elements containing TRE and/or GRE half-sites arranged as direct repeats (GG, TG, GT, and TT) (Table I). We also tested the abilities of these TRs to transactivate reporter plasmids containing these hybrid response elements. Our results suggest that two half-sites are required for TR/TRAP heterodimer binding to DNA, but heterodimer can form when both two half-sites are present only on one of the half-sites. We also found that the sequential arrangement of strong (TRE) and weak (GRE) half-sites in the TRE may be a critical determinant for TR/XR heterodimer binding to the TRE and transcriptional activation.

**Materials and Methods**

Preparation of In Vitro Translated Receptors—Previously described cDNA clones of rTRα in pSC (35), rNRα in pBS (13) (kindly provided by Dr. K. Ozato, National Institutes of Health, Bethesda, MD), were used in these experiments. TR-P box was created by changing the 3 amino acids of the P box on the first zinc finger of TRα (EGRK) to that of the GR (EGRK) by using a sense strand oligonucleotide (36) containing three-point mutations specifying new codons: CGCCTGTACCTGCTGAGCTGCACTTGCTTCGCCG, TRα in pSC5, and the Clontech in vitro mutagenesis kit (Clontech, Palo Alto, CA). The mutations in the P box region of the TR-P box mutant cDNA were confirmed by DNA sequencing. Each cDNA was linearized with the appropriate restriction endonuclease and used as a template for RNA synthesis with 

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\text{RNA synthesis with T7 RNA polymerase. Unlabeled and } [\text{35S}]\text{-methionine-labeled receptors then were produced from rabbit reticulocyte lysates according to the manufacturer's instructions (Life Technologies, Inc.). Unprogrammed reticulocyte lysate also was incubated under the same conditions. Translated } [\text{35S}]\text{-methionine-labeled receptor protein was quantitated by trichloroacetic acid precipitation (8, 10), and by SDS-polyacrylamide gel electrophoresis analysis which showed labeled proteins of expected molecular weights. Both TRα and TR-P box proteins had similar binding to } [\text{32P}]\text{J}
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Preparation of Nuclear Extract—Nuclear extract from CV-1 cells were prepared and stored as described previously for 235–1 rat pituitary cells (8).

**Design of Oligonucleotide Probes**—Deoxyribonucleotides containing TRE or GRE half-sites arranged as direct repeats with a four nucleotide gap were used in our experiments. GG, GT, TG, and TT (Table I). The half-sites were bounded by four nucleotides that were identical to the TRE and GRE half-sites. HindIII and XhoI sites were placed on either end to gap nucleotides. HindIII and XhoI sites were placed on either end to gap nucleotides. HindIII and XhoI sites were placed on either end to gap nucleotides. HindIII and XhoI restriction sites on 5' and 3' ends were cloned into the EcoRI and BamHI sites of rat pituitary plasmid (36). These reporter plasmids then were sequenced to ensure that only a single copy of the hybrid response element had been incorporated.

CV-1 cells were grown in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum. The serum was stripped of T3 by constant mixing with 5% (v/v) AG1-X8 resin (Bio-Rad) twice for 12 h at 4 °C before ultrafiltration. The cells were transfected with expression plasmids as well as a Rous sarcoma virus β-galactosidase control plasmid (3 µg) unless otherwise indicated (39) in 6-cm plates using the calcium-phosphate precipitation method (40). Cells were stained with 45 h in the absence or presence of 10^{-3} M T3, before harvesting. Cell extracts were analyzed for both luciferase (41) and β-galactosidase (39) activity.

**Results and Discussion**

We first analyzed TR-P box binding to hybrid response elements containing either TRE or GRE half-sites arranged as a direct repeat with a four nucleotide gap sequence (Table I). The nucleotide sequences that bounded the half-site sequences were identical to the gap sequences so that we could assess the orientation of TR complex binding to these response elements without potential confounding effects by different flanking sequences. TR-P box could not bind as a monomer or homodimer to any of the hybrid response elements Fig. 1A). It was unable to bind as a TR-P box/XR heterodimer to GG or GT; however, surprisingly, TR-P box/XR heterodimer bound to TG and TT. These results suggest that TR-P box/XR heterodimer selectively bound to a response element in which the GRE half-site was the downstream half-site. Moreover, heterodimerization with RXR permitted TR-P box binding to TG and TT when it was unable to bind to these response elements alone. Additionally, TR-P box/XR heterodimer unexpectedly bound to TT per-

| Table I: Nucleotide sequences of hybrid response elements and single half-sites |
|:---|:---|
| Response element | Sequence |
| GG | CATGAGGACCATGAGGACCATAG |
| TG | CATGAGGTCACATGAGGACCATAG |
| GT | CATGAGGTCACATGAGGACCATAG |
| TT | CATGAGGTCACATGAGGACCATAG |
| GmT | CATGAGGACCATGAGGACCATAG |
| TGm | CATGAGGTCACATGAGGACCATAG |
| GTm | CATGAGGTCACATGAGGACCATAG |
| TmG | CATGAGGACCATGAGGACCATAG |

Yale University) or rRXRα in pCMV (kind gift of M. G. Rosenfeld, University of California, San Diego), and 50,000 cpm of methylated labeled probe were performed and the heterodimer bands and free probe bands excised. The DNA was isolated, treated with 1 M PIPES for 30 min at 90 °C, purified, lyophilized, and suspended in formamide loading buffer, before being subjected to electrophoresis.
hapt due to RXR interaction with the TRE half-site. Addition of 10^-7 M T₃ did not affect the pattern of TR-P box binding to these response elements but did increase slightly the mobility of TR-P box/RXR heterodimer binding to TG and TT (data not shown).

We next analyzed TRα binding to these hybrid response elements (Fig. 1B). TRα did not bind to GG but was able to bind to GT, TG, and TT as a monomer. TRα also bound weakly to TT as a homodimer similar to our previous observations with DR4 (18). In contrast to TR-P box/RXR heterodimer binding to these response elements, TRα/RXR heterodimer bound to GT, TG, and TT with TRα/RXR heterodimer binding to TT > TG ≥ GT. Additionally, we also examined TRα/TEAP and TR-P box/TEAP heterodimer formation on these response elements using CV-1 nuclear extract and found that they had a similar pattern as TRα/RXR and TR-P box/RXR heterodimer binding to these sequences (data not shown). Indeed, recent immunodepletion experiments in our laboratory using anti-RXR antibodies suggest the major TRAP in CV-1 cells is RXRβ or a related protein (16).²

Our data showing TRα/RXR heterodimer binding to GT and TG, and TR-P box/RXR heterodimer binding to TT, suggested that the heterodimer could bind to elements containing at least one strong half-site. However, it is not known whether TR/RXR heterodimer binding requires two half-sites or could occur on only one half-site with protein/protein interactions alone stabilizing the heterodimer. Accordingly, we mutated either the GRE (TGm and GmT) or TRE (TmG and TmG) half-sites of TG

² P. M. Yen, M. Ikeda, E. C. Wilcox, J. H. Brubaker, R. A. Spanjaard, A. Sugawara, and W. W. Chin, unpublished results.
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**Fig. 4. Methylation interference studies of TRα/RXRβ heterodimer binding to TG and GT.** A, sequences of TG and GT. Coding sequence is on the top and noncoding sequence is on the bottom. The 5' end is on the left for the coding sequence and on the right for the noncoding sequence. B, fluorographs of methylation interference experiments of TRα/RXR heterodimer binding to TG and GT. Labeling of TG and GT fragments and methylation interference studies of TRα/RXR heterodimer binding to TG and GT were performed as described under "Materials and Methods." Methylation interference was quantitated by laser densitometry (Molecular Dynamics, Sunnyvale, CA) and normalized to the intensity of labeled probe at the top of the gel. Note that dimethyl sulfate methylates guanine residues at the N-7 position and weakly methylates adenines at the N-3 position. Abbreviations: striped arrow, downstream half-site; dark arrow, upstream half-site; •, complete or near complete methylation interference (>80%); Δ, partial methylation interference (39% for TG coding and 35% for GT noncoding nucleotides after normalization); F, free probe; B, bound TR/RXR heterodimer.

and GT and examined TRα and TR-P box complex binding to these response elements. TRα monomer bound to GmT and TGm as expected but could not form TRα/RXR heterodimers on these elements (Fig. 2A). TRα could not bind as a monomer to TmG or GTm (Fig. 2B), demonstrating that TR monomer binds to the TRE, but not the GRE, half-site. Additionally, TRα/RXR
heterodimer could not bind to TmG or GTm. TR-P box monomer or heterodimer could not bind to any of these mutated sequences (Fig. 2, A and B). Taken together with the previous findings (Fig. 1), these results suggest that both half-sites are required for TR/RXR heterodimer formation and that at least one of these half-sites must be a strong half-site for TR/RXR heterodimer binding.

We next examined the ability of TR-P box and TRa to mediate T3-dependent transcriptional activation in co-transfection experiments using reporter plasmids containing the hybrid response elements. TR-P box was unable to stimulate transcription from any of the reporter plasmids in the presence 10^{-4} M T3 (data not shown). These results suggest that although TR-P box can form heterodimers on TG and TT, they are not transcriptionally active. In contrast, TRa was able to stimulate transcription from TG and TT, but was either unable, or minimally able, to stimulate transcription from GG and GT (Fig. 3). Thus, although TR/RXR heterodimer can bind to GT, TG, and TT, it is much more active when bound to the latter two elements.

In order to examine further the mechanism for the difference in T3-mediated transcriptional activation via TG and TT, we studied the methylation interference patterns of TR/RXR heterodimer binding to these elements (Fig. 4). Both half-sites were occupied by TR-P box heterodimer, further confirming that two half-sites are required for heterodimer binding. Additionally, greater methylation interference of guanine residues was observed in the upstream half-site of TG, whereas the opposite was observed for GT. These results suggest that TRa/RXR bound to these elements in different orientations and/or distinctly different conformations.

In the foregoing studies, we have examined the DNA-binding and transcriptional activity of TR-P box and TRa on hybrid response elements containing GRE and TRE half-sites. TR-P box/RXR heterodimer was able to bind to TG but not GT, suggesting that the arrangement of half-sites in a direct repeat can dictate whether this heterodimer can bind to the hybrid response element. Additionally, these data suggest that TR-P box may bind to the downstream half-site of TG. Recently, while this work was in progress, two other groups used a similar approach to demonstrate that heterodimers containing P box mutants of TR8 and RXRa prefer binding to the downstream and upstream half-sites, respectively (42, 43); however, the functional significance of either mutant or wild-type heterodimer binding to the hybrid response elements was not examined. At present, it is not known whether wild-type TR/RXR has a similar orientation requirement when bound to direct repeats. However, the ability of TR-P box monomer to bind only to the TRE half-site, and the differences in TRa/RXR heterodimer methylation interference patterns on TG and TT, suggest that TRa/RXR heterodimer may bind in different orientations on the hybrid response elements.

We also have shown that TRa/RXR heterodimer formation on the hybrid response elements requires both half-sites since mutating even a weak half-site (GRE half-site) abolishes TRa/RXR heterodimer binding to DNA. Methylation interference studies further confirmed that TRa/RXR was able to contact both TRE and GRE half-sites of a hybrid response element. However, TR/RXR heterodimer interaction with at least one strong half-site (TRE half-site) is required for heterodimer binding to DNA since TRa/RXR can bind to GT and TT but not GG.

We also showed that formation of TR/TRAP heterodimers on hybrid response elements may be necessary but not sufficient for T3-mediated transcriptional activation. TR-P box/RXR heterodimer bound to TG and TT but was transcriptionally inactive, suggesting that P box amino acid substitutions can affect transcriptional activity either directly or indirectly via conformational changes. In this respect, it recently has been shown that the second amino acid of the P box (glycine) may be important for T3-mediated transcription (44). Interestingly, a mutant TR in which the entire DNA-binding domain was replaced with the GR DNA-binding domain also was transcriptionally inactive on these response elements (32).2 TRs/RXR bound to GT, TG, and TT, but was transcriptionally active only on TG and TT, suggesting that TRa/RXR bound to GT may not be in the optimal orientation or conformation for transcriptional activation.

Previously, it has been shown that nucleotide spacing and orientation of TRE half-sites, as well as flanking sequences, can influence TR binding and transcriptional activation (5, 25, 26, 29, 45). Our studies of TR-P box and TRa on the hybrid response elements strongly suggest that arrangement of upstream and downstream half-sites in the direct repeat motif can influence TR/RXR and TR/TRAP heterodimer binding to DNA. Such arrangement of half-sites can have functional consequences since the orientation and/or conformation of ligand TR/TRAP heterodimer on a TRE may be critical determinants of its ability to transactivate on that TRE. We speculate that the nucleotide sequence degeneracy observed among natural TRE half-sites can result in the sequential arrangement of strong and weak half-site sequences, which, in turn, may play an important role in modulating T3-mediated transcriptional activation.

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