Thyroid Hormone Alters *in Vitro* DNA Binding of Monomers and Dimers of Thyroid Hormone Receptors

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T_3 binds to intranuclear thyroid hormone receptors (TRs) on target DNA elements and exerts profound influences on gene expression by mechanisms not yet characterized. We used gel shift assays and cross-linking experiments to demonstrate that T_3 greatly induced the monomeric binding of the hTRβ produced in *Escherichia coli* to DNA. T_3 also increased the gel mobility of these monomer-DNA complexes suggesting they undergo a ligand-induced conformational change. This effect did not depend on the orientation and spacing of the half-site motifs within the DNA structure. In contrast, T_3 had diverse effects on the dimeric interaction. T_3 increased the dimeric interaction to the palindrome GGTCA·TGACC (an effect lost by spacing the half-sites with 3 base pairs) and decreased the dimeric interaction to the inverted palindrome containing the TGACC·GGTCA motif. Scatchard analyses indicated that the T_3 enhancement on binding was due to an increase in the number of TR with high affinity DNA-binding activity and not by increasing the affinity of TR that could bind to DNA. The effects of various T_3 analogs were directly related to their affinities for the TR. These ligand effects on *in vitro* TR-DNA binding may reflect mechanisms by which T_3 regulates transcription *in vivo*. (Molecular Endocrinology 6: 1142–1152, 1992)

INTRODUCTION

Thyroid hormone receptors (TRs) are members of the steroid/thyroid superfamily of intracellular receptors (1). These proteins consist of an assembly of modular structures or domains engaged in diverse but somewhat overlapping biological functions (2–6). The DNA binding domain allows the receptor to recognize responsive elements usually localized in the promoter region of regulated genes, while the ligand binding domain binds specifically and with high affinity the cognate ligand.

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In intact cells, the unliganded steroid receptors are present in the cytoplasm or in the nucleus free of chromatin (7) and are associated with heat-shock proteins (hsp) including hsp 90 (8). Upon ligand binding, the receptors dissociate from hsp90 and adopt their DNA-binding properties that were masked by the hsp90 interaction. Ligand binding to steroid receptors appears to promote dimerization (9, 10), thereby enabling the receptor subunits to exhibit positive cooperativity in their DNA binding to palindromic sites (11, 12). Also, ligand binding may affect the gel mobility of steroid receptor-DNA complexes (13, 14).

In contrast to steroid receptors, the TRs do not associate with hsp90 (15) and are tightly bound to chromatin in the absence of hormone (16). These unliganded TRs can influence gene expression *in vivo* when bound to specific TR-binding sites (TREs) (17). Thus, the model has evolved that the major active thyroid hormone form, T_3, meets the TR already bound to DNA and provokes a change in the TR-DNA complex that activates its transcriptional regulatory properties (18). The molecular mechanisms of T_3 action are, however, largely unknown. Several TR isoforms have been reported: TRα1 (19, 20); TRβ (20, 21); TRβ2 (22); and one isoform, TRα2, that does not bind T_3 (23). *In vitro* studies performed with rat liver TR (24), with recombinant TRα1 (25–27), and with recombinant TRβ (25, 28) showed that addition of T_3 had no effect on TR binding to DNA. However, a T_3 inhibitory effect on the binding of TRα1 (29) and TRβ (27, 29) to DNA has been recently reported (27), as well as a T_3 disruptive effect on receptor homodimer binding to DNA (29). T_3 inhibition of TR homodimers but not of heterodimers (29) are in agreement with recent reports that suggest that heterodimers of TR with retinoid X receptors (RXR α or β) are required for effective TR-DNA interaction (30–34). However, the diversity of functional TREs that include palindromes, inverted palindromes, and direct repeats with various spacings and sequence degeneracy within the half-sites (28, 35), suggest that depending on DNA context TR may bind and regulate gene expression as monomers, homodimers, or heterodimers. It is also possible that different configurations of dimers (head-

head, head-tail, and tail-tail) bind different DNA structures with different effects.

We report here that binding of bacterially produced human TRβ (hTRβ) to a variety of TREs is highly influenced by T_3 and analogs of T_3. Ligand binding markedly augments the maximum amount of TRs that bind DNA as monomers. By contrast, T_3 may have positive or negative effects on the dimeric TR-DNA interaction depending on the orientation of the half-sites within the DNA structure. In addition, T_3 may induce a conformational change on the monomeric TR-DNA interaction as evidenced by an increased gel mobility of these complexes. These hormone-induced changes in the TR-DNA interaction may influence the way TR regulates transcription.

RESULTS

Human TRβ Expressed in *Escherichia coli* Binds Specifically to a Variety of TREs as Both Monomer and Dimer

We used a gel shift assay to assess the specificity of the binding of partially purified hTRβ expressed in *Escherichia coli* to the DNA-containing TREs shown in Fig. 1A. The first oligonucleotide, TREpal, a variant of a rat GH TRE (36), contains the palindromic motif GGTCA·TGACC and *in vivo* confers positive regulation by T_3. The second oligonucleotide is derived from the natural sequence of the estrogen response element in the *Xenopus* vitellogenin A2 gene (−328/−289; vitA2) and contains the same palindromic motif except for the presence of 3 base pairs at the center of dyad symmetry. The third oligonucleotide is a natural sequence from the chicken embryonic myosin gene (−31/−1; EM1) that binds with high affinity the native rat liver TR (37); this sequence contains one perfect TGACC half-site and a second imperfect one (Fig. 1A, *bracketed*). The fourth oligonucleotide (EM1pal) is a mutated version of EM1, with two EM1 half-sites in a dyad symmetry but in an opposite orientation (inverted palindrome) to those found in the TREpal and vitA2 oligonucleotides. The fifth oligonucleotide (glucocorticoid response element; GRE), used as a control, is a palindromic variant of the consensus binding site for the glucocorticoid receptor (2).

Figure 1B shows that, in the absence of T_3, bacterially expressed hTRβ forms one major and one minor band with the [^{32}P]EM1 probe. We interpreted the major lower complex as a monomer and the upper minor complex as a dimer TR-DNA interaction (see below). As indicated, all of the nonlabeled TRE-containing oligonucleotides competed with the radiolabeled probe for the formation of the dimer and monomer complexes, whereas an equivalent amount of unlabeled GRE did not compete. The DNA structures TREpal and vitA2, with two half-sites in the GGTCA·TGACC orientation, competed more effectively than EM1, which has only one half-site, and EM1pal, in which the half-sites are in the opposite orientation.

Several investigators have interpreted upper and lower bands observed in gel shift assays as corresponding to monomeric and dimeric TR-DNA interactions (29, 38–41). Williams *et al.* (41) argued that only the upper band (dimers) showed positive cooperativity in their DNA binding, and Lazar *et al.* (40) showed that mixing wild type with an amino-terminally deleted form of TRβ yields a novel complex (heterodimer) that migrates between the upper but not between the lower bands, therefore indicating that the upper bands were due to homodimerization of wild type and of truncated TRβ. Although these results are consistent with monomers and dimers, the stoichiometry of these TR-DNA complexes has not been established. To address this issue, we performed the gel shift assays in low melting agarose and cross-linked the material present in the upper and lower TR-DNA complexes *in situ* by exposing the gel to UV irradiation (see Fig. 4, lanes 2, 5, 8, and 11, to observe the pattern of the upper and lower bands formed with the various TREs). Figure 2 shows the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the cross-linked products of the various bands. As indicated by the *arrows*, the lower bands using [^{32}P]EM1 [59 kilodaltons (kDa)], vitA2 (61 kDa), and TREpal (53 kDa) DNAs resulted only in molecular size species corresponding to a TR monomer (52 kDa) plus DNA. By contrast, the upper bands (from EM1pal and TREpal) revealed approximately 114-kDa complexes that correspond to a dimer of TR plus DNA. No other bands were revealed by overexposing the gels (17 days).

Although the cross-linking studies demonstrate the stoichiometry of upper and lower bands of TR-DNA interactions, they did not rule out that the dimer complexes might merely represent independent binding of two monomers. To address more rigorously this issue, we examined the pattern of binding by varying the DNA concentration. As seen in Fig. 6B (lanes 1–7), vast excess of EM1pal DNA (30 half-sites per one TR) could not convert upper (dimeric) into lower (monomeric) complexes. These results are incompatible with independent binding of monomers to form the upper band and indicate the two TRs do interact to bind DNA. In the EM1pal case, the linear Scatchard plot suggests that the two TRs bind as a unit (form dimers in solution) rather than bind cooperatively to DNA. Similarly, dimeric binding to TREpal was enhanced and not abolished (see Fig. 6C) by vast excess of DNA, but in this case the TR-TR interaction seemed much weaker than with EM1pal.

These results demonstrate that the bacterially expressed hTRβ binds specifically to DNA-containing TREs as both monomer and dimer, and that the monomer formation is favored with the EM1 DNA.

T_3 and T_3 Analogs Promote the Interaction between TR and DNA

To address whether thyroid hormone ligands that bind to the TR influence the pattern of binding of the bacte-

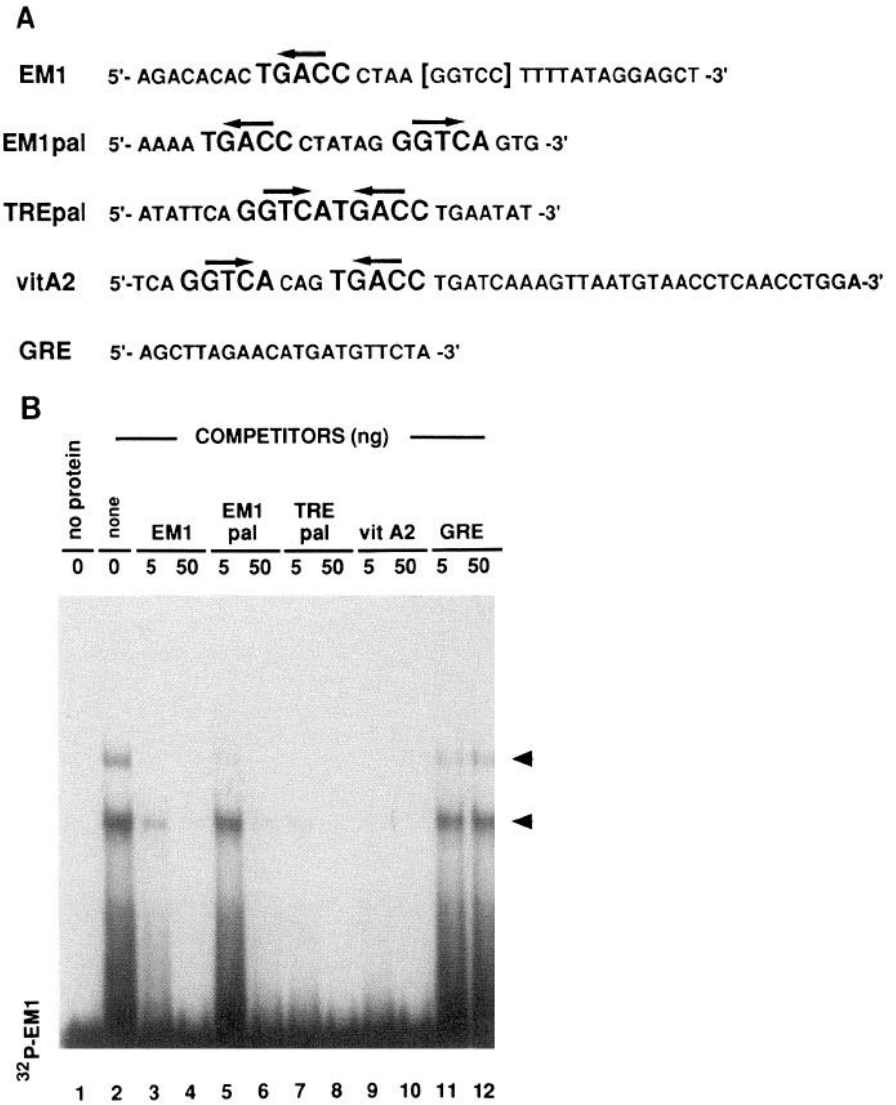

Fig. 1. Specific Binding of the Bacterially Expressed hTRβ to a Variety of TR Binding Sites

A, Sequence of the oligonucleotides used in the assay. The putative monomer binding site is represented in *bold type*. The imperfect half-site of EM1 is *bracketed*. The *arrows* represent the orientation of the half-sites. B, Gel shift assay of the TR-DNA interactions. Binding assays contained 160 fmol partially purified (7%) hTRβ expressed in *E. coli* and 10,000 cpm (1 fmol) [^{32}P]EM1 probe. For competition experiments the probe was incubated with the receptors in the absence (lane 2) or presence of 5 and 50 ng unlabeled EM1 (lanes 3, 4), EM1pal (lanes 5, 6), TREpal (lanes 7, 8), vitA2 (lanes 9, 10), and GRE (lanes 11, 12). The *solid arrows* indicate the specifically bound TR-DNA complexes.

rially expressed TR to the EM1 DNA, we performed binding studies in the absence and in the presence of increasing amounts of T_3 and T_3 analogs (Fig. 3).

Binding of the unliganded hTRβ resulted in a predominant monomer and a faint dimer TR-EM1 complex (Fig. 3, lane 1). The monomer band is actually two bands that are due to the two molecular size species in the partially purified preparation used in these studies. A T_3 concentration-dependent (Fig. 3, lanes 2–6) enhancement in the formation of both monomer TR-DNA bands was observed, and the effect appeared to be greater on the lower molecular size species, although quantitative analyses were not performed. Ligand binding also completely inhibited the faint dimer complex and increased slightly the mobility of the stimulated monomer complex. Addition of 10^{-6} M 3,5,3′-triiodothyroacetic acid (Triac), which binds these receptors more tightly than T_3 [dissociation constant (K_d) ~0.12 nM; Apriletti, J. W., B. L. West, and J. D. Baxter, in preparation], stimulated the formation of the monomer complex to an even greater extent than did maximally effective concentrations of T_3 (Fig. 3, lane 7). T_4, which binds to these receptors more weakly than T_3 (K_d ~20 nM; Apriletti, J. W., B. L. West, and J. D. Baxter, in preparation), added at 10^{-5} M also stimulated, albeit less effectively (Fig. 3, lane 8), the TR-DNA interaction.

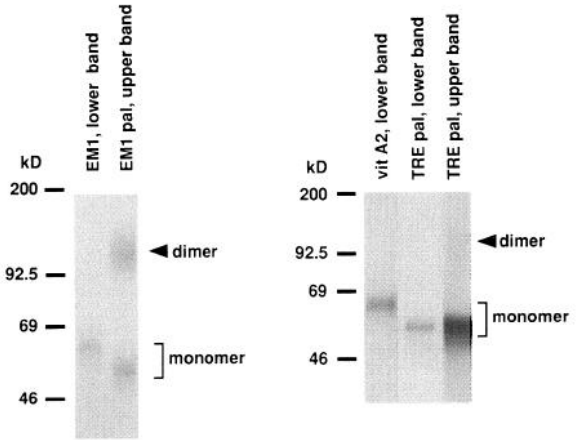

Fig. 2. Cross-Linking *in Situ* of Upper and Lower TR-DNA Bands Reveals Monomers and Dimers of TR

Binding reactions using 200,000 cpm [^{32}P]EM1, 200,000 cpm [^{32}P]EM1pal, 300,000 cpm TREpal, and 650,000 cpm vitA2 were incubated each with 800 fmol bacterially produced hTRβ purified to greater than 98%. The TR-DNA complexes were resolved in a 1.5% low melting agarose gel. The upper bands of the TR-EM1pal and of the TR-TREpal complexes and the lower bands of the TR-EM1, TR-TREpal and TR-vitA2 complexes were UV cross-linked *in situ*, excised, melted in SDS, and subjected to SDS-PAGE analysis.

Addition of 10^{-5} M reverse T_3 (r-T_3) which binds weakly to these receptors (K_d ~300 nM, Apriletti, J. W., B. L. West, and J. D. Baxter, in preparation), caused an even lesser effect (Fig. 3, lane 9). Thus, under these conditions, all of the T_3 agonists enhance bacterially expressed TR binding to DNA, but the magnitude of the effect varies according to the ligand, suggesting there is a correlation between the affinity of the ligand for binding TR and the extent to which the ligand can stimulate the TR binding to DNA.

It is possible that the T_3 effect could simply be due to preventing protein degradation. We ruled out this possibility by changing the order of addition of T_3. Reactions preincubated without T_3 for 10, 15, and 18 min and subsequently exposed to T_3 for 10, 5, and 2 min display similar T_3 enhancement effects (data not shown).

T_3 Enhancement Effect on TR-DNA Binding Is Observed with a Variety of DNA Structures

To examine whether the hormone stimulation on TR-DNA interaction was dependent on the nature of the DNA, we tested the binding of the bacterially expressed hTRβ to the other three specific TREs listed in Fig. 1A. As shown in Fig. 4, T_3 again enhanced formation of the monomer TR-EM1 complex and decreased the barely perceptible dimer complex (lanes 2 and 3). In contrast, in the absence of T_3, the TR bound to the EM1pal oligonucleotide (inverted palindrome) as a prominent dimer and a barely perceptible monomer (Fig. 4, lane 5). T_3 markedly enhanced the monomer and slightly

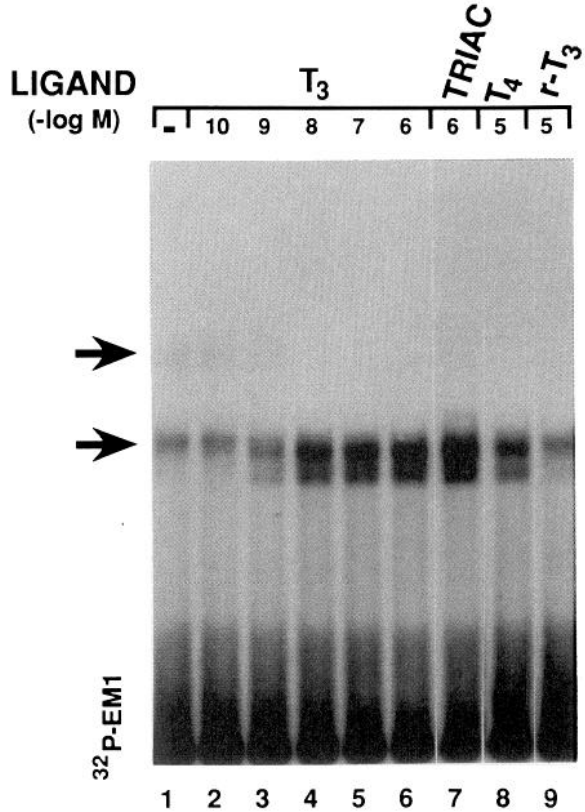

Fig. 3. T_3 and T_3 Analogs Promote Binding of Bacterially Expressed hTRβ to the Chicken Embryonic Myosin (−31/−1) Oligonucleotide (EM1)

Partially purified (7%) hTRβs expressed in *E. coli* (90 fmol) were incubated with 5,000 cpm [^{32}P]EM1 (2 fmol) oligonucleotide for 20 min at 22 C. Lane 1 shows the retarded TR-DNA complex formed in the absence of T_3. Lanes 2–6 show the reactions in the presence of increasing concentrations of T_3. The effect of different T_3 analogs are shown for Triac (1 μM; lane 7), T_4 (10 μM; lane 8), and r-T_3 (10 μM; lane 9). *Solid arrows* point to the prominent monomer and to the faint dimer TR-DNA complex. Notice that T_3 treatment inhibits the dimer and enhances the mobility of the monomer band.

diminished (28% as measured by laser densitometry) the dimer band (Fig. 4, lane 6).

The TREpal and vitA2 structures, both of which exhibit the half-sites in the orientation originally proposed as the consensus TRE (GGTCA·TGACC), gave a different pattern of TR-DNA interaction. Both the monomer and dimer complexes are readily apparent (Fig. 4, lanes 8 and 11). T_3 again greatly enhanced the monomer bands with both the TREpal and vitA2 probes, but the effects on the dimer bands differed. T_3 stimulated the formation of the dimer with the TREpal probe (Fig. 4, lane 9) but had no major effect (slightly inhibitory in most experiments) on the formation of the dimer band with the vitA2 probe (Fig. 4, lane 12).

These results demonstrate that the DNA structure influences both the pattern of TR binding to DNA and the way that T_3 influences the binding. T_3 increases the monomer TR-DNA formation in all cases, and depend-

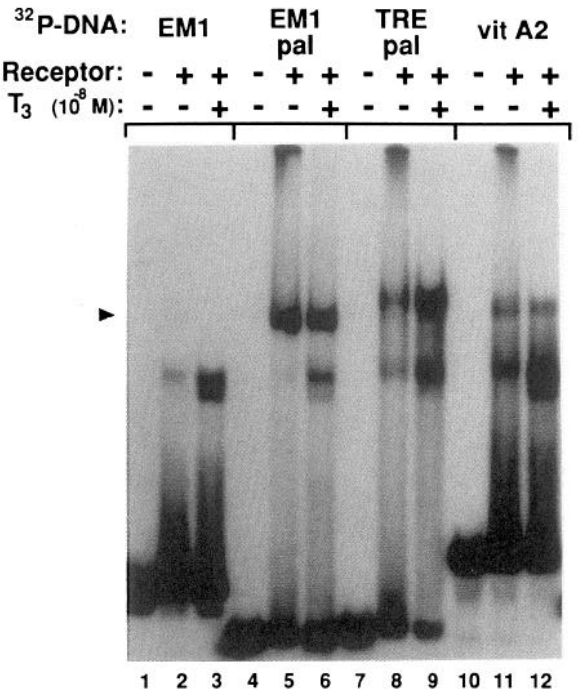

Fig. 4. T_3 Promotes Binding of Bacterially Expressed hTRβ to a Variety of DNA Structures

Gel shift assays of TR-DNA interaction. Binding assays contained 80 fmol partially purified (7%) hTRβ expressed in *E. coli* and 10,000 cpm [^{32}P]EM1 (1.5 fmol). Lanes 1, 4, 7, and 10 show the unbound probes. Retarded receptor-DNA complexes formed in the absence of T_3 are shown in lanes 2, 5, 8, and 11. Retarded receptor-DNA complexes formed in the presence of 10 nM T_3 are shown in lanes 3, 6, 9, and 12. The *solid arrow* points to the barely perceptible dimer complex present with the EM1 probe in the absence of T_3.

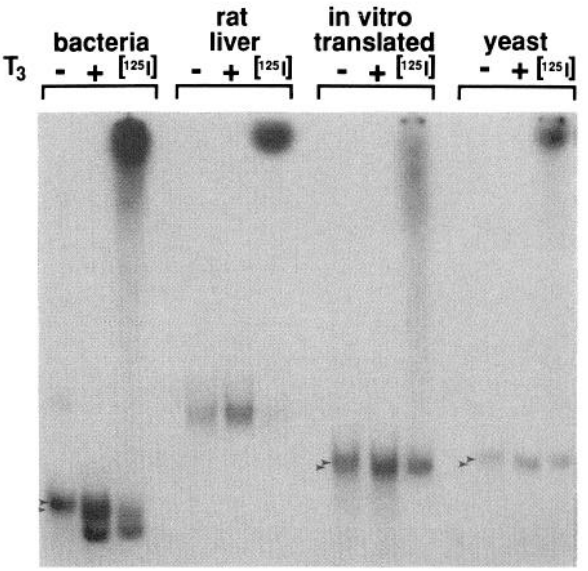

Fig. 5. T_3 Enhances the Binding of T_3 Receptors Produced in Eukaryotic Sources to the EM1 Oligonucleotide

Different receptor preparations were incubated with 5000 cpm [^{32}P]EM1 probe (1 fmol) for 20 min at 22 C in the absence or presence of 10 nM T_3. The free probe was run off the gel. Binding reactions using 90 fmol hTRβ expressed in *E. coli* are shown in lanes 1–3. Lane 1 shows the migration of the complex formed by unlabeled receptors and [^{32}P]EM1 in the absence (lane 1) or presence (lane 2) of T_3. Lane 3 shows the complex formed by [^{125}I]T_3-labeled TR and 10 ng nonradioactive EM1. Binding reactions using the partially purified (1%) rat liver receptor (7 fmol) are shown in the absence (lane 4) or presence (lane 5) of T_3. Lane 6 shows the complex formed by [^{125}I]T_3-labeled rat liver TR and 10 ng nonradioactive EM1. Binding reactions using 15 fmol hTRβ, synthesized in reticulocyte lysate, are shown in the absence (lane 7) or presence (lane 8) of T_3. Lane 9 shows the complex formed by [^{125}I]T_3-labeled *in vitro* synthesized TR and 10 ng nonradioactive EM1. Binding reactions using 10 fmol partially purified (1%) hTRβ expressed in yeast are shown in the absence (lane 10) or presence (lane 11) of T_3. Lane 12 shows the complex formed by [^{125}I]T_3-labeled yeast TR and 10 ng nonradioactive EM1. *Arrowheads* mark the differences in migration caused by T_3 treatment.

ing on the DNA element, T_3 selectively affects the proportion of receptors that bind as monomers and dimers.

T_3 Alters the DNA Binding Pattern of TRs Produced in Other Systems

We next examined whether T_3 could also influence DNA binding of TRs produced in other systems where the presence of auxiliary proteins and/or postranslational modifications might alter the DNA-binding properties of these receptors.

Figure 5 shows that binding of the EM1 probe to TR translated in reticulocyte lysate, produced in yeast, or purified from rat liver is also affected by T_3. There was a clear and reproducible T_3 enhancement on TR-DNA interaction using TR from all preparations, although the T_3 enhancement effect with these TRs was less pronounced than that observed with the bacterially expressed TR. Figure 5 also shows results with [^{125}I]T_3-labeled TR that demonstrate the presence of TR proteins in the retarded complexes.

In addition to increasing TR-DNA binding, T_3 also increased slightly, but reproducibly, the mobility of the monomer TR-DNA complexes formed with TR produced in bacteria, lysate, and yeast (indicated by the *arrowheads* in Fig. 5). Although this effect is not evident in Fig. 5 with the rat liver TR bound to the EM1 probe, it was observed in other experiments with the rat liver TR bound to the vitA2 DNA (data not shown).

The migration of TR-DNA complexes varied according to the source of TR. The complexes formed with rat liver TR were more retarded than the ones formed with the various recombinant hTRβs due to their association with an uncharacterized nonreceptor protein (40, 42; data not shown). The recombinant hTRβ produced in reticulocyte lysate or yeast formed TR-DNA complexes with identical gel migration. In contrast, the gel migration of monomers with bacterially recombinant hTRβ was much faster. These differences in gel mobility among the various TR-DNA complexes maybe due to

the lack of posttranslational modifications on TRs produced in bacteria.

These results indicate that T_3 enhances the overall DNA binding of TR produced in several different systems and provokes conformational changes on TR-DNA complexes, and suggest that the presence of factors or of posttranslational modifications in eukaryotic preparations may induce TRs to have some T_3-independent DNA-binding activity not seen in bacterial TR.

T_3 Increases the Number of TRs That Can Bind DNA

The marked T_3 enhancement on the binding of bacterially expressed TR to various DNA structures (Fig. 6) could result from a change in the affinity of the receptor for DNA or from a change in the number of receptors able to bind DNA. To address this issue, saturation experiments similar to the ones described by Fawell *et al.* (43) were conducted in which the amount of TR-DNA binding was measured with increasing DNA concentrations.

The results of these experiments employing a constant amount of hTRβ expressed in *Escherichia coli* and ^{32}P-labeled DNAs are shown in Fig. 6. To achieve high concentrations of DNA, we diluted a fixed amount of the particular radioactive probe with increasing amounts of unlabeled DNA and corrected accordingly the specific activity in calculations (43). Bound and free complexes were quantified directly using storage phosphor technology. The advantages of storage phosphor over film autoradiography are a linear dynamic range covering five orders of magnitude and a sensitivity up to 250 times that of film (see Ref. 57). The data for monomer and dimer interactions were analyzed separately. The TR-DNA binding increased with increasing concentrations of DNA and approached saturation at the highest DNA concentrations used with EM1pal (Fig. 6B), TREpal (Fig. 6C), and vit A2 (Fig. 6D), but not with the EM1 (Fig. 6A) DNA.

Scatchard plots with the EM1pal and vitA2 probes could be interpreted as a straight line and a single bimolecular reaction, but the plots with the EM1 and TREpal probes did not conform to a single straight line, suggesting two classes of receptor-DNA interaction. Nonetheless, the extrapolated values from the Scatchard plots indicate that, independent of DNA structure, T_3 markedly augmented the number of receptors that bind DNA as monomers without changing the slopes of the plots. On the other hand, T_3 had DNA-dependent effects on the dimer interaction. T_3 decreased the total number of TR that formed dimers with the EM1pal probe and had no major effect on the slope of the plot. Note that the quantified T_3 effect on EM1pal dimers is modest but reproducible and cannot be appreciated by visual inspection of the exposure shown in Fig. 6B. T_3 increased the total number of TRs that formed dimers with the TREpal probe and again had no major effect on the slope of the plot. The slopes of the dimer interaction with the EM1 and vitA2 probes and of the monomer interaction in the absence of T_3 with the EM1pal probe could not be determined, because there was either no TR-DNA binding (EM1pal monomer in the absence of T_3) or the binding could not be detected when increasing DNA concentration. The loss of binding at higher DNA concentrations imply that these dimer interactions (EM1 and vitA2) are not stable or might merely represent independent binding of two monomers. Similar results, although quantitatively less pronounced, were obtained in identical studies that analyzed the T_3 effects on saturation binding of *in vitro* translated hTRβ to these various oligonucleotides (data not shown). Altogether, these results indicate that the dominant effect of T_3 is to influence the number of TRs that bind as monomers or dimers rather than to change their affinity for the various DNAs.

The data also imply that there is heterogeneity in the receptor preparations. This possibility is suggested by the nonlinear Scatchard plots with the EM1 and TREpal probes and by the great difference in the maximal receptor binding to the various DNAs (compare maximum binding in Fig. 6, A–D). The greatest binding (5.4 nM) is achieved by dimers on EM1pal, whereas binding of dimers to TREpal is much lower (0.2 nM). The monomer binding (0.1–1.1 nM range) is also widely diverse among the various DNAs and is lowest for the EM1 that did not appear to saturate the receptor binding capacity at the highest DNA concentration used. Thus, there appears to be a larger population of TR in these preparations that can bind to the EM1pal structure than to the other DNA structures, and the most prominent T_3 influence is to increase the number of receptors that can bind as monomers.

In summary, T_3 had no major effect on the affinity of bacterial TR for DNA. T_3 increased the maximal amount of TRs that form monomers with all tested DNA structures, increased the maximal binding of dimers to DNA with the GGTCA·TGACC orientation and unspaced half-sites, and decreased the maximal binding of dimers to DNA with the half-sites in the inverted orientation.

DISCUSSION

The results of this study demonstrate that T_3 can greatly influence the way TR recognizes DNA *in vitro*. T_3 binding to partially purified bacterially expressed hTRβ increased the monomeric TR-DNA interaction, had varied effects on the dimeric interaction, and increased the mobility of the monomer TR-DNA complexes.

The T_3-enhancement effect on DNA binding was dose dependent, reaching maximal stimulation around 10^{-8} M T_3. Interestingly, Triac, which has a higher affinity than T_3 for TR, produced an even greater stimulation than the maximally effective concentrations of T_3. By contrast, T_4, which binds to the TR with about 5% of the affinity of T_3, was at saturating concentrations less effective than T_3, and r-T_3, the ligand with weakest

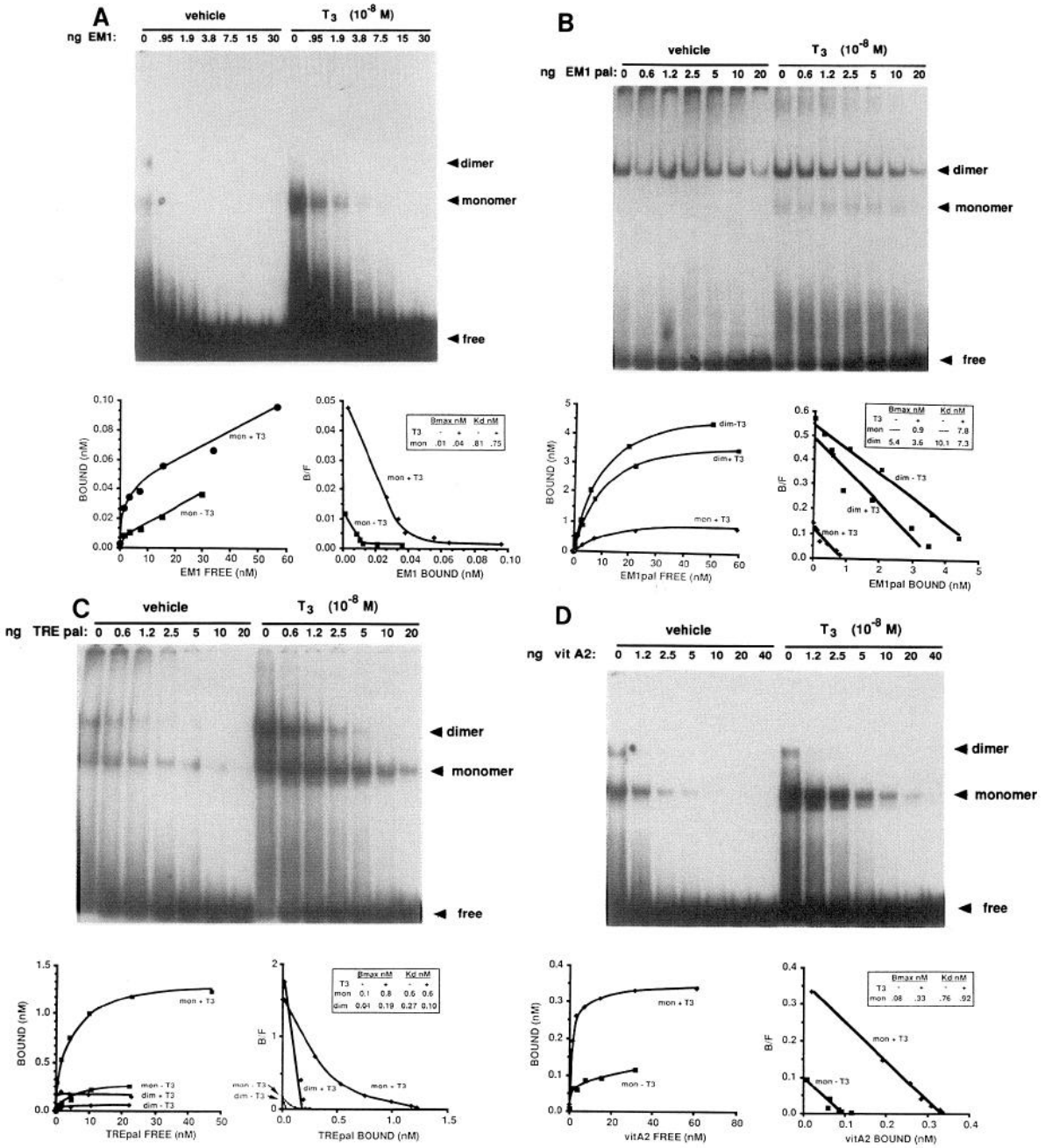

Fig. 6. T_3 Augments the Maximum Amount of Bacterially Expressed hTRβ Bound to DNA as Monomers and Depending on the DNA Structure Selectively Alters the Formation of Dimer TR-DNA Complexes

Saturation experiments using the gel shift mobility assay to estimate binding parameters of TRs to a variety of DNA structures. Partially purified (7%) TRs expressed in *E. coli* (90 fmol) were analyzed for DNA binding activity in the absence or presence of 10 nM T_3 over the range of DNA concentrations indicated. Increasing amounts of DNA were achieved by diluting the labeled probe with unlabeled oligonucleotide and correcting accordingly the specific activity in calculations (43). Radioactivity associated with free DNA and with receptor-DNA complexes was then quantified directly by placing the dried gels on phosphostimulable storage phosphor-imaging plates. After exposure at room temperature, the imaging plates were scanned with a 10 milliwatt helium-neon laser by means of a galvanometer-controlled mirror. The resulting digital image was then viewed on a video monitor and analyzed using Molecular Dynamics (Sunnyvale, CA) ImageQuant software (57). Quantified bound and free complexes were corrected according to the diluted specific activity, and saturation binding analyses were performed using a nonlinear iterative curve fitting program prepared to be used with IGOR (Wavemetrics, Lake Oswego, Oregon). The curves and Scatchard plots generated from the analysis of the monomer and dimer TR-DNA binding are shown immediately below the x-ray film autoradiography of the correspondent gel shift assay. The estimates of the maximum bound (Bmax) and of the affinity (K_d) are shown in the *insets* of the Scatchard plots. A, Saturation experiment using the [^{32}P]EM1 oligonucleotide (12,000 cpm, ~2.4 fmol) and increasing amounts of unlabeled EM1. B, Saturation experiment using the [^{32}P]EM1pal oligonucleotide (6,100 cpm, ~1.6 fmol) and increasing amounts of unlabeled EM1pal. C, Saturation experiment using the [^{32}P]TREpal oligonucleotide (14,000 cpm, ~2.2 fmol) and increasing amounts of unlabeled TREpal. D, Saturation experiment using the [^{32}P]vitA2 oligonucleotide (15,000 cpm, ~2.9 fmol) and increasing amounts of unlabeled vitA2.

affinity for TR, was at saturating concentrations the least effective of all tested ligands. These results could imply that in addition to their affinities, these ligands also differ in their intrinsic abilities to provoke DNA-binding activity. If these results are confirmed by functional studies, they would imply that the major active thyroid hormone, T_3, is a partial and not a full agonist. In this regard, there are indications in the literature that these compounds may differ in their intrinsic agonistic activities (44).

The most pronounced effects of T_3 were with TR produced in bacteria. T_3 did augment DNA binding of TR produced in reticulocyte lysate, expressed in yeast, and extracted from rat liver. However, the magnitude of the stimulation was lower than that observed with bacterially synthesized TR, and this may account for the fact that such stimulation was not previously reported. It is possible that mammalian/eukaryotic factors that associate with or modify the TR, to promote a tighter TR-DNA interaction, might be absent in the bacterial preparations. Several investigators have described auxiliary proteins that promote TR-DNA binding in a T_3-independent manner (25, 45, 46), and most recently several groups have identified the RXRs as one group of these proteins (30–34). The RXRs heterodimerize with TR and both enhance *in vitro* TR binding to TREs and increase T_3-induced transcriptional activity on TRE-containing promoters (30–34). It is interesting to note that binding of bacterial TR to the EM1-DNA was enhanced by its heterodimerization with the rat liver auxiliary protein, and addition of T_3 could further stimulate this heterodimer formation (data not shown). Furthermore, the native rat liver receptors employed in the experiment shown in Fig. 5, also appears to be a heterodimer of TR and a nuclear factor (data not shown) and this heterodimer was stimulated by T_3. In summary, these findings suggest that auxiliary proteins and/or posttranslational modifications within the eukaryotic preparations may alter the DNA-binding properties of TR and explain the diminished T_3 stimulation observed in this study on the binding of endogenous rat liver, reticulocyte lysate, and yeast TRs to DNA.

Our saturation studies suggest that the stimulation was due to an increase in the number of receptors that bind to DNA rather than an increase in the affinity of the TR. Therefore, in the starting mixture, there are at least two functionally distinct classes of receptors, activated receptors that bind specifically to DNA, and nonactivated receptors that do not bind DNA in the absence of T_3. Apparently, the bacterial preparation contains a population of TRs capable to bind T_3 but not DNA (nonactivated), and after binding T_3 these TRs adopt a DNA-binding conformation (activated). This model assumes that some portion of the receptors in the starting preparation was in the activated state by T_3-independent mechanisms. One could envision an allosteric equilibrium between the activated and nonactivated TRs and that T_3 and possibly other factors shift the equilibrium toward the activated form.

The T_3 enhancement effect on monomers was accompanied by an increment in the gel mobility of these complexes, whereas the mobility of dimers was unaltered by T_3. These findings suggest that ligand occupation modifies the conformation of monomers and that the increased mobility of these complexes is not a fortuitous effect caused by the presence of T_3. Similar ligand-induced increased mobility was recently reported for the interaction between the vitamin D receptor, another member of the nuclear receptor superfamily, and its DNA-responsive element (47).

The use of different DNA structures revealed that the presence of palindromic motifs and the orientation and spacing of their half-sites influences the nature of the TR-DNA interactions, and that addition of T_3 alters the pattern of such interactions. These core motif-dependent differences in monomer *vs.* dimer formation may reflect the fact that switching the orientation of TR on the DNA exposes novel dimerization interfaces that differ in their ability to self-associate, and our results indicate that T_3 may exert a differential influence on the formation of these interfaces. The only nonpalindromic DNA (EM1) showed prominent monomer and weak dimer bands, whereas its palindromic version (EM1pal) showed exactly the opposite composition of TR-DNA bands. Addition of T_3 decreased dimer formation and promoted the monomer interaction. These results are also in agreement with those of Yen *et al.* (29), which also showed that T_3 inhibited binding of TR homodimers to DNAs with the TGACC·GGTCA configuration of half-sites.

With DNAs containing the GGTCA·TGACC orientation, both the monomer and dimer complexes are readily apparent. T_3 also enhanced the binding of monomers to these structures, but unlike the DNA with the half-sites in the inverted orientation, T_3 enhanced the dimer TREpal complex and had minor effects on the dimer vitA2 complex. The importance of the orientation and spacing of half-sites has been recently emphasized as a means of selecting transcriptional response to T_3, retinoic acid, and vitamin D (28, 35). Our findings that the T_3 effect is largely dependent on the architecture of the half-sites suggest a potential hormone-mediated mechanism through which distinct DNA structures might give rise to unique transcriptional responses.

In view of the findings with estrogen and progesterone receptors that ligand-binding induces dimerization, the finding that T_3 predominantly enhances the formation of monomers is surprising and fundamentally different from the mode of action of steroid receptor ligands. It is noteworthy that unlike many of the binding sites for steroid receptors, the known naturally occurring TREs such as those in the rat GH (48), human chorionic somatomammotropin (49), and malic enzyme (50) genes correspond more to nonpalindromic motifs. Recently, direct repeats of half-sites have been identified in naturally occurring genes (28, 35), and it would be interesting to investigate TREs containing direct repeats with a variety of spacings.

Monomerization may constitute an interesting alternative for gene regulation. It was recently reported that

ligand binding to AraC protein derepresses transcription by breaking dimeric bonds between two AraC proteins. These proteins now reassociate using new dimerization interfaces and occupy a previously unoccupied half-site on DNA (51). It would be plausible to find similar mechanisms controlling T_3-responsive genes. We hypothesize that T_3, depending on promoter/cell context, can generate transcriptionally active monomers or, alternatively, can generate monomers that readily adopt novel homodimer configurations or establish heterodimers such as the ones described with accessory proteins (25, 40, 45, 46), retinoic acid receptor (52), and other members of the nuclear receptor superfamily (30–34). By promoting different monomeric, homodimeric, or heterodimeric configurations, T_3 can greatly expand the combinatorial possibilities of regulating gene expression.

MATERIALS AND METHODS

Reagents

T_3 was purchased from Aldrich (Milwaukee, WI); r-T_3, T_4, and Triac were obtained from Sigma (St. Louis, MO).

In Vitro Transcription and Translation

An expression plasmid, pT7T319U (Pharmacia, Piscataway, NJ), containing the cDNA encoding the hTRβ was linearized at a downstream-engineered *Cla*I site, and capped RNA was synthesized (Promega Riboprobe System, Madison, WI) using T7 RNA polymerase. RNA (1–2 μg) was translated in a rabbit reticulocyte lysate (Promega) containing 40 μM $ZnCl_2$, a methionine-free amino acid mixture (Promega), and 20 μM cold methionine. The size of the translated protein was determined by performing parallel reactions in the presence of ^{35}S-labeled methionine and checking the products by SDS-PAGE.

Rat Liver TRs

Partially purified TRs from rat liver (1%) were prepared as described previously (53).

TR Expressed in Bacteria

The hTRβ was expressed in *E. coli* using a T7 promoter and isopropyl-thiogalactopyranoside induction and purified as described elsewhere (Apriletti, J. W., B. L. West, and J. D. Baxter, in preparation). Briefly, TRs were solubilized from the bacteria by lysozyme treatment followed by sonication and partially purified by polyethyleneimine and ammonium sulfate precipitation steps. Approximately 30 mg soluble receptor at 0.2% purity was obtained from a 50-liter fermentor run. Four major T_3 binding species were obtained from the bacteria. The full length receptor (52 kDa) and a 47-kDa fragment (missing 4–5 kDa from the amino-terminal end) comprise the two DNA-binding forms, whereas two smaller fragments (33 and 35 kDa) are missing the entire DNA-binding domain and bind to heparin weakly. The relative proportions in crude extract were approximately: 52 kDa, 50–70%; 47 kDa, 5–15%; 35 kDa, 15–30%; and 33 kDa, 5–15%. The receptor was loaded onto a phenyl-Toyopearl hydrophobic interaction column at 0.5 M ammonium sulfate and eluted with a descending linear gradient from 0.5–0 M ammonium sulfate. The receptor peak was diluted 2-fold with water, loaded onto a TSK-Heparin HPLC column (TosoHaas, Philadelphia, PA), and eluted with a 50–400 mM NaCl gradient. The full length receptor and the 47-kDa fragment eluted at 0.28 M NaCl and was approximately 7% pure, whereas the two smaller non-DNA-binding fragments eluted at 0.15 M NaCl. These receptors bind T_3 with an affinity of 0.35–1.4 nM (Apriletti, J. W., B. L. West, and J. D. Baxter, in preparation) that is similar to the one reported for the human placental TRβ (54).

TR Expressed in Yeast

The hTRβ was expressed in *Saccharomyces cerevisiae* using the YEPE2 expression vector and the BJ3505 yeast strain as recently described for the production of the estrogen receptor (55). TRs were solubilized from the yeast by vortexing with glass beads and partially purified by polyethyleneimine treatment and ammonium sulfate precipitation. The final extract was approximately 1% pure.

T_3 Binding Assay

Receptor concentration was determined as previously described (53). The binding assay contained the receptor sample to be assayed, 1.5 nM [^{125}I]T_3, 50 μg/ml core histones, 5 μg/ml for each of the four protease inhibitors (antipain, leupeptin, pepstatin and chymostatin), and Buffer A (20 mM potassium phosphate, pH 7.6, 0.4 M KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol, and 0.1% monothioglycerol) in a 0.25-ml vol reaction. Nonspecific binding was measured by adding 1000-fold excess of unlabeled T_3. After overnight incubation at 4 C, 200 μl of the reaction was loaded on a Quick-Sep Sephadex G-25 column equilibrated with Buffer A. The excluded peak of protein-bound [^{125}I]T_3 was eluted with 1 ml Buffer A, collected in a test tube, and counted. Specific T_3 binding was calculated by subtracting nonspecific binding from total binding.

Gel Shift Binding Assay

TR binding to DNA was assayed by gel retardation analysis (24). The TR-DNA complex was visualized either by labeling DNA with ^{32}P using polynucleotide kinase or by using TRs bound with [^{125}I]T_3. For [^{125}I]T_3 labeling, TRs were incubated with 0.75–1.5 nM radioactive T_3 for 60–120 min at 4 C. The same incubation procedure was used to expose various concentrations of unlabeled T_3 and T_3 analogs to TRs in order to study the effect of hormone on TR-DNA interaction. TRs were mixed with various DNA concentrations, typically 1–5 fmol for [^{32}P]DNA or 20–1300 fmol for unlabeled DNA, and 2 μg poly dI-dC (Pharmacia LKB) in a 20-μl vol reaction. The binding buffer contained 10 mM $NaPO_4$, 1 mM $MgCl_2$, 0.5 mM EDTA, 20 mM NaCl, 5% glycerol, 0.1% monothioglycerol, and 5 μg/ml of each of the protease inhibitors. After 20 min at 24 C, the mixture was loaded onto a 5% nondenaturing polyacrylamide gel that was previously run for 30 min at 200 V. To separate the TR-DNA complexes, the gel was run at 4 C for 120–240 min at 240 V using a running buffer containing 6.7 mM Tris-base (pH 7.5 for a 10× stock at room temperature), 1 mM EDTA, and 3.3 mM Na acetate.

TR-DNA Cross-Linking Assay

We slightly modified a protocol (56) in which the cross-linking procedure is performed after resolving the TR-DNA complexes in a gel shift assay. This method allows individual protein-nucleic acid complexes to be cross-linked *in situ*, visualized by autoradiography, excised, and analyzed by SDS-PAGE. Briefly, we incubated 200,000–650,000 cpm ^{32}P-labeled DNA and 800 fmol of a preparation of hTRβ purified to near homogeneity (>98% as judged by silver staining; Apriletti, J. W., B. L. West, and J. D. Baxter, in preparation) as described in the gel shift binding assay, in a 13–15 μl vol reaction. To obtain such a high degree of purification, chromatography of the receptor saturated with T_3 was used in the final step of

purification, and this procedure rendered these TRs unsuitable for studies evaluating the T_3 effects on their DNA binding. After 20 min at 24 C, the reactions were loaded onto a 0.5-cm-thick horizontal 1.5% low melting agarose gel in 6.7 mM Tris-base (pH 7.5 for a 10× stock at room temperature), 1 mM EDTA, and 3.3 mM Na acetate and run at 4 C for 2.5–3 h at 120 V. The TR-DNA binding patterns were identical to the ones observed in polyacrylamide gels. After running, the gel was placed over Saran Wrap on the filter surface of a 310-nm UV transilluminator at 4 C, irradiated for 10 min, and then autoradiographed for 20 min. The upper and lower bands were excised and weighed. To each 50 μl melted gel slice we added approximately 15 μl of a mixture of 0.3 M Tris-HCl, pH 6.8, 6% SDS, 15% glycerol, and 70 mM dithiothreitol, and boiled the samples for 3 min. The warm melted samples were loaded on 1.5-mm-thick empty wells and the complexes separated by (4% stacking-8% resolving) SDS-PAGE.

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