Nucleic Acids Research, Vol. 20, No. 18 4803–4810

Thyroid hormone alters the DNA binding properties of chicken thyroid hormone receptors α and β

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Received June 25, 1992; Revised and Accepted August 17, 1992

ABSTRACT

The effects of thyroid hormone agonists on thyroid hormone receptor (TR)/DNA complex formation was investigated to elucidate the mechanism by which TRs transactivate genes in response to ligand. The data, obtained from gel shift experiments, indicate that thyroid hormones alter the conformation of TRs bound to DNA, irrespective of if the element is occupied by monomeric TR, homodimeric TR/TR, or heterodimeric complexes with the retinoid receptors RAR or RXR. Furthermore, triiodo-thyronine (T3) prevents 2 TR molecules from binding to oligonucleotides containing direct repeats or inverted palindromes of the consensus AGGTCA motif, an effect that was not detected with palindromic elements. Heterodimers bound to direct repeats were less affected: RXR/TR were fully and RAR/TR complexes partially resistant to thyroid hormone. The data suggest that a ligand-induced conformational change in TR prevents double TR occupancy of a response element containing 2 direct repeats of the consensus binding motif, possibly by steric hindrance, whereas such an event does not prevent TR/RXR heterodimers from binding to DNA. Finally, our data show that a monomeric, liganded TR bound preferentially to the second half site in a AGGTCActcaAGGTCA element, and therefore indicate that nucleotides adjacent to the consensus half site contribute to binding specificity.

INTRODUCTION

Steroid and thyroid hormone receptors induce transcription in presence of ligand by binding to specific sequences representing hormone response elements located in the control region of a target gene [1]. For the estrogen-, progesterone and glucocorticoid receptors the ligand is required for dissociation of the receptor from the hsp90 complex, dimerization of the receptor molecules, and for binding to DNA. Several agonists and antagonists affecting receptor/DNA interaction and transcriptional activation have been reported [2–6]. The estrogen receptor (ER) agonist estradiol, which allows DNA binding and transcriptional activation, induces a conformational change in the receptor as shown by a faster migration of the receptor/DNA complex in polyacrylamide gels [5]. Tamoxifen, a partial ER agonist that allows the receptor to bind to response elements, does not permit the receptor/ligand complex to transactivate. That tamoxifen does not cause the ER/DNA complex to migrate faster, suggests that a conformational change in the ER is necessary for transcriptional activation. Moreover, the ER ligand ICI 164.384 functions as a pure antagonist by preventing the receptor complex from binding to the response element [7], possibly by interfering with receptor dimerization [5]. In contrast, the progesterone antagonist RU486 allows the progesterone receptor to bind a response element and induces a conformational change in the receptor, but does not induce transcription of progesterone regulated genes [3, 6].

The receptors for vitamin D3 (VDR), thyroid hormone (TR) and retinoic acid (RAR) differ from those for estrogen, progesterone, and glucocorticoid in that they bind with high affinity to response elements consisting of repeated motifs of the recognition sequence AGGTCA, spaced by 3, 4 or 5 nucleotides respectively [8–10]. In addition, DNA binding and transactivation can take place from a synthetic response element representing a palindrome with no spacing between the consensus half sites [11].This group of receptors transactivate genes by forming heterodimers with 9-*cis*-retinoic acid receptors [12–18] or other proteins [19, 20]. However, at high receptor concentration 2 TR molecules readily bind to oligonucleotides containing 2 consensus half sites [21, 22].

To elucidate the mechanism by which TRs transactivate genes in response to ligand, we studied the effects of thyroid hormone agonists [23] on receptor/DNA complex formation. The results from gel shift experiments suggest that triiodothyronine (T3) alters the conformation of TRs binding to DNA as monomers, homodimers, or heterodimers. T3 also prevents 2 TRs from forming homodimeric complexes on oligonucleotides containing direct repeats or inverted palindromes of the consensus motif AGGTCA, although this was not the case with palindromic elements. In contrast, TR/RXR heterodimers readily bound to palindromic elements in the presence of T3, although also these complexes had an altered mobility.

The data demonstrate that a ligand-induced conformational change in TR prevents double TR occupancy of some response

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elements, possibly by steric hindrance, whereas such an event does not occur with heterodimeric complexes. Finally, our finding that both liganded and unliganded TRs bind preferentially to the second half site in a response element consisting of 2 direct repeats spaced by 4 nucleotides suggests that nucleotides adjacent to a binding site influence binding affinity.

MATERIALS AND METHODS

Receptor preparations

Recombinant Vaccinia viruses were used for overexpression of receptors as described previously [22, 24–26]. Briefly, HeLa cells were infected at high multiplicity of infection, and nuclear extracts prepared 16 h p.i. The levels of receptor were estimated by analysis of [^{35}S]-Methionine labelled nuclear extracts in SDS polyacrylamide gels and by [^{125}I]-T3 binding assay [27]. Similar amounts of receptor (~1% of total nuclear protein) were found in the extracts regardless of the type of receptor expressed.

cDNAs encoding the following receptors were expressed with Vaccinia vectors: full length (46kD) chicken TRα [28]; TRα-p40 that represents an N-terminally truncated form of cTRα and which lacks 2 phosphorylation sites present in the full length receptor [22, 29]; cTRβ0 [30]; the oncogenic P75$^{gag\text{-}v\text{-}erbA}$ protein that does not bind ligand [27, 31]; the V2-erbA recombinant in which the defective ligand binding domain was substituted for that of cTRα [27]; hRARα; and hRXRα.

Oligonucleotides

Five μg of single stranded, complementary, oligonucleotides (purchased from Scandinavian Gene Synthesis, Köping, Sweden) representing natural response elements of direct or palindromic repeats were denatured at 95°C in a waterbath, and then annealed in 180 mM NaCl by slow cooling overnight. The resulting HindIII ends were labelled in a fill in reaction with the Klenow fragment of DNA polymerase I.

Gel retardation assay

Gel retardation assays were done as described previously [22]. The incubation buffer contained 4% Ficoll, 60 mM KCl, 5 mM $MgCl_2$, and 10 mM Hepes pH 7.9. Carrier poly d(I)d(C) DNA was added to a final concentration of 0.1 μg/μl for reactions containing oligonucleotides and 0.2 μg/μl when DNA fragments were used. In binding reactions approximately 60 nM of receptor was used, usually equivalent to 3 μl of Vaccinia virus extract, in a total volume of 20 μl. All incubations were done on ice, and polyacrylamide gels were run at 4°C. The ligands used in binding studies were: T3 = 3,3',5 -Triiodo-L-thyronine, T2 = 3,5 Diiodo-L-thyronine (Sigma D0629), T4 = L-Thyroxine (Sigma T2376), rT3 = 3,3',5'-Triiodo-L-thyronine (Sigma T0781) and Triac = 3,3',5-Triiodo-Thyroacetic acid (Sigma T7650). Saturation curves and Scatchard plots for TR/DNA complexes where done as described [22].

Shift-western blotting

Essentially, the procedure of Demczuk et al was used (manuscript in preparation). Receptor-containing nuclear extracts were mixed with unlabelled Dir 4 or Pal 0 oligonucleotide and a tracer amount of [^{125}I]-T3 was added, followed by addition or not of increasing concentrations of unlabelled T3. The mixture was electrophoresed under gel shift conditions followed by electrotransfer to 2 different membranes placed on top of each other. The first filter (nitrocellulose) specifically retained the proteins whereas the second (DEAE) the radiolabelled ligand. The protein filter was subsequently developed with rabbit anti-cTRα antibodies, and both filters exposed to film.

Methylation interference assays

For DNA methylation interference assays the Dir 4 oligonucleotide was cloned into the pBL2 vector [32], and a 114 nucleotide fragment encompassing the oligonucleotide was labelled by PCR amplification. The resulting fragment, labelled in one end, was purified in polyacrylamide gels, partially methylated [33], and incubated with nuclear extract containing unliganded cTRα. T3 was then added to half of the cTR/DNA complex, and both samples loaded on gel retardation gels after 10 min. Retarded bands were excised, and the purified fragments partially cleaved with piperidine followed by analysis in denaturing polyacrylamide gels.

Quantification

A BioDynamics Phosphor Imager and the software ImageQuant were used to quantify the radioactivity in the gel retardation assays.

RESULTS

T3 affects TR/DNA complex formation

The effects of ligand on the DNA binding properties of several chicken thyroid hormone receptors were tested. For this, we overexpressed two forms of TRα (TRα and TRα-p40), and TRβ0 using Vaccinia virus vectors (fig 1A). Nuclear extracts containing the TRα or TRb receptors were incubated with T3, and the ligand-saturated receptor preparations were allowed to bind a synthetic oligonucleotide (Dir 4), representing a direct repeat of the AGGTCA consensus motif spaced by 4 nucleotides (fig 1B).

Gel retardation assays (fig 2A) showed that in the absence of ligand two complexes were formed, representing one (1×TR) or two molecules (2×TR) binding to the oligonucleotide [22]. In contrast, prior addition of T3 allowed formation almost exclusively (>95%) of complexes containing only one receptor molecule, irrespective of the receptor tested. Moreover, the T3 treated 1×TR complexes migrated faster in the gel, indicating that the receptors had undergone a conformational change upon binding of ligand. Similar results (data not shown) were obtained with a similar oligonucleotide representing a natural thyroid hormone response element from the MuLV LTR [24]. Control experiments with wild type Vaccinia extracts gave no retarded bands (Fig 2A, lane C).

To test whether the T3 effect was dependent on the spacing between the half sites, we examined the binding of TRα to the oligonucleotides Dir 0, Dir 2, RAREβ and Dir 6 (fig 1B) in which the half sites are separated by 0, 2, 5, or 6 nucleotides, respectively. The RAREβ represents a retinoic acid receptor response element [10, 34]. An oligonucleotide containing only one half site was also tested (denoted 'half-site'). The analyses (fig 2B) show that in the absence of hormone, TRα binds almost exclusively as a monomer to the elements 'half site' and Dir 2, whereas the other oligonucleotides allowed significant amounts of 1×TR and 2×TR complexes to form. Addition of T3 to the extracts inhibited 2×TR complex formation on the Dir 0 and the RAREβ elements but not on the Dir 6 oligonucleotide. In all instances the hormone caused the TR/DNA complexes to migrate faster.

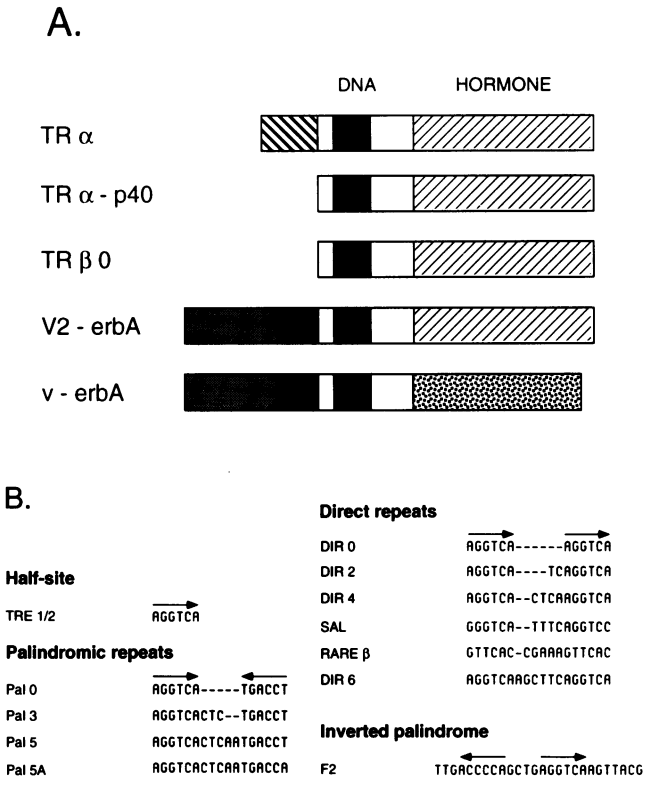

Figure 1. Comparison of chicken thyroid hormone receptors and oligonucleotides used in this study. (A) TRα-p40 is 36 amino acids shorter than TRα in the N-terminal domain, and lacks two phosphorylation sites present in the longer receptor. TRβ0 is similar to TRα-p40 in overall structure, with ~85% amino acid similarity. (B) Organisation of consensus AGGTCA half sites in the oligonucleotides used.

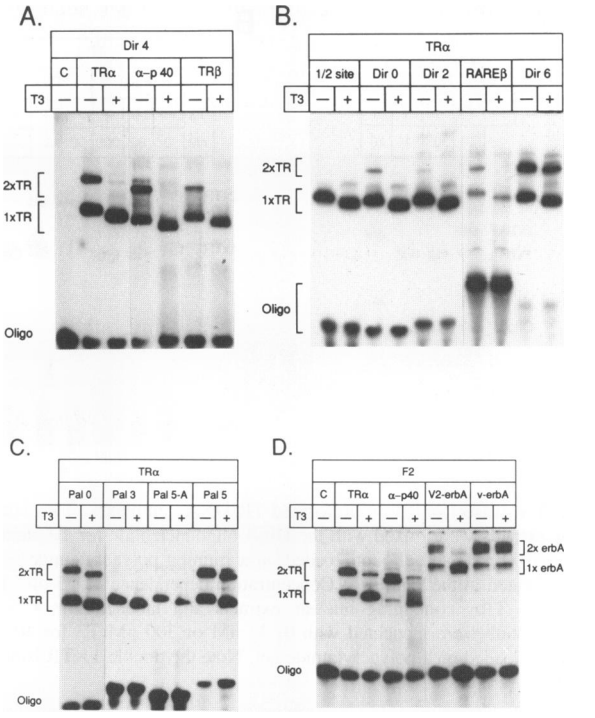

Figure 2. Effects of T3 on DNA binding. TR-containing nuclear extracts from Vaccinia vector infected cells were incubated for 10 min with 100 nM T3, labelled oligonucleotides were added for 10 min, and the resulting protein/DNA complexes analysed by gel retardation assays. Binding of T3-bound **a)** TRα, TRα-p40 and TRβ0 to the Dir 4 oligonucleotide, **b)** TRα to direct repeat elements, **c)** TRα to palindromic elements, and **d)** TRα and v-erbA related proteins to inverted palindromes. The sequences of the oligonucleotides used are given in Figure 1B and in the methods section.

Next, we tested the binding of TRα in the presence and absence of T3 to elements harbouring the half sites in a palindromic fashion. In these, the half sites were separated by 0, 3, or 5 nucleotides (fig 1B); consequently, the Pal 3 oligonucleotide represents an idealised estrogen response element [35]. The gel retardation assays (fig 2C) show that the hormone allowed two receptor molecules to bind to the Pal 0 and Pal 5 elements, whereas oligonucleotides Pal 3 and Pal 5-A (in which one half site was mutated to TGACCA) bound only one receptor molecule irrespective of the presence of ligand. Again, the TR/DNA complexes migrated faster after treatment with ligand.

Finally, we tested the effect of T3 on the binding of TRα isoforms to the F2 thyroid hormone response element from the chicken lysozyme gene, in which the palindromic half sites are organised in the reverse order [36]. The results show that T3 inhibited most of the 2×TR complex formation with both normal TRα tested. To verify that binding of ligand is a prerequisite for the T3 effect, we incubated extracts containing the v-erbA protein with T3 prior to addition of the F2 oligonucleotide. The v-erbA protein is an oncogenic variant of TRα that has lost the ability to bind T3, and contains 251 amino acids of the retroviral gag polypeptide in the N-terminal domain [37]. The gel retardation analysis (fig 2D) shows that T3 had no effect on 2×TR formation. A control protein V2, in which the defective ligand binding region of v-erbA was replaced with the corresponding region of TRα, only bound one receptor molecule. Similar results were obtained with a natural response element from the MuLV LTR [24], representing 2 direct repeats spaced by 4 nucleotides [8].

To test whether T3 also could dissociate preformed 2×TR complexes, T3 was added to TRα/DNA complexes formed on the Dir 4 element, and aliquots analysed after increasing time of incubation. Figure 3A shows that double receptor occupancy of the elements was abolished within 30 s of incubation.

To determine the concentration of ligand required for dissociating the 2×TR complexes and for causing an increased mobility, nuclear extracts were incubated with increasing amounts of hormone. Figure 3B shows that with both the Pal 0 and the Dir 4 oligonucleotides, 30 nM T3 caused ~50% of the protein/DNA complexes to migrate faster. 300 nM T3 inhibited all 2×TR complex formation on the Dir 4 element, but had no effect on the same complex on the Pal 0 oligonucleotide.

A direct demonstration that T3 binds to receptor/DNA complexes was achieved by a shift-western blot (Demczuk et al, manuscript in preparation). Accordingly, receptor-containing nuclear extracts were mixed with unlabelled Dir 4 or Pal 0 oligonucleotide and tracer amount of [^{125}I]-T3 added, followed by addition or not of increasing concentrations of unlabelled T3. The mixture was electrophoresed under gel shift conditions followed by electrotransfer to 2 different membranes placed on top of each other. The first filter specifically retained the proteins whereas the second the radiolabelled ligand. The protein filter was subsequently developed with anti-cTRα antibodies, and both filters exposed to film. Figure 4 shows that the antibodies detected receptor proteins in 1×TR and 2×TR complexes (right panels A and B), and that partial saturation of the receptors with T3

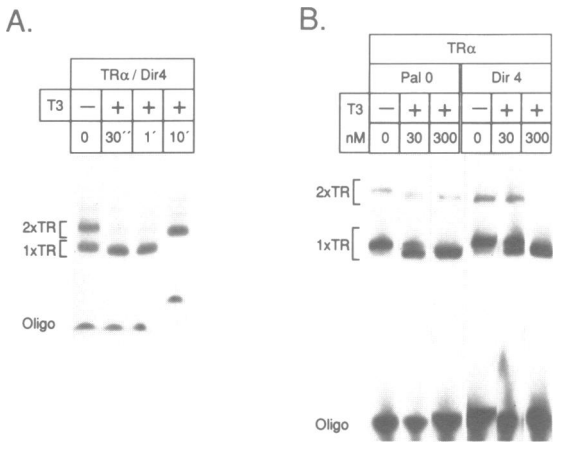

Figure 3. a) Effect of T3 on preformed TR/DNA complexes. TRα-containing nuclear extracts were mixed with the Dir 4 oligonucleotide for 10 minutes, T3 added to 100 nM, and aliquots loaded on a running polyacrylamide gel at the times indicated in the figure. **b)** Concentration dependence of T3 on TR/DNA complexes. TRα containing nuclear extracts and labelled Dir 4 or Pal 0 oligonucleotide were incubated with 0, 30 nM or 300 nM T3 for 10 minutes and finally loaded on a polyacrylamide gel. Note the double 1×TR bands at the 30 nM T3 samples.

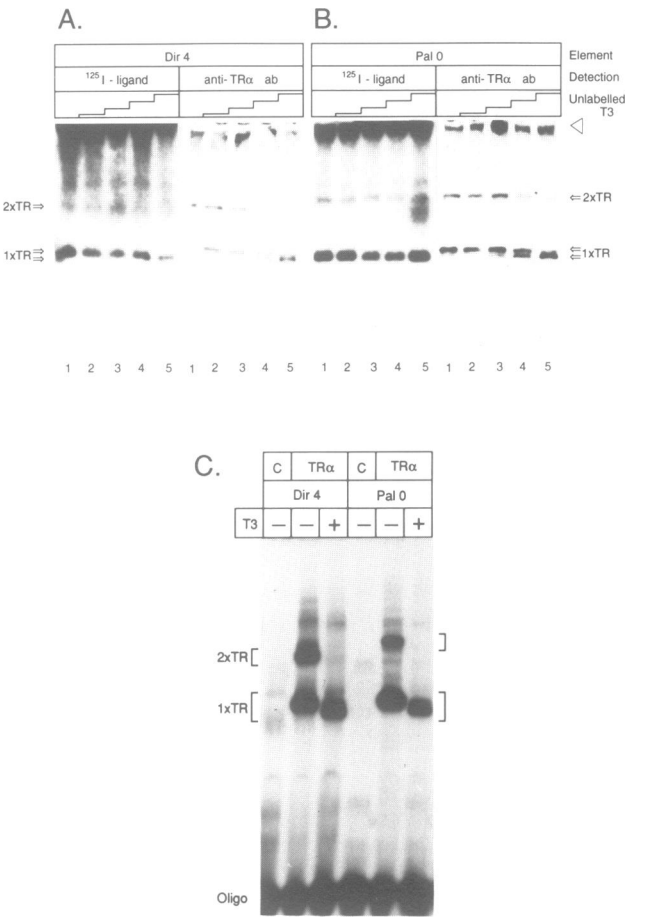

Figure 4. Detection of T3 in receptor/DNA complexes. TRα and **a)** Dir 4 or **b)** Pal0 were mixed with tracer amounts of labelled T3 and with increased amounts of unlabelled T3 and electrophoresed under non-denaturing conditions. Lane 1 contains 0, Lane 2 = 2.7 nM, lane 3 = 8.0 nM, lane 4 = 20 nM and lane 5 = 80 nM of unlabelled T3. Left panels show T3 in receptor/DNA complexes. Right panels show antibody-detected TRα that was electro-transferred to a nitrocellulose filter from the same band shift gel. **c)** DNA binding capacity of TRα saturated in vivo with T3. TRα-expressing Vaccinia vector infected cells were incubated with medium containing T3 or T3-depleted serum during the course of infection. Nuclear extracts prepared from these cells were incubated with the Dir 4 or Pal 0 elements, and analysed on polyacrylamide gels.

caused the 1×TR complexes to migrate faster. Radiolabelled T3 (left panels) was found both in the 1×TR and 2×TR complexes of Pal 0, whereas only the 1×TR complex on the Dir 4 element contained labelled ligand. A third, slowly migrating complex containing [^{125}I]-T3 was also observed. This complex represents TRα/RAR or RXR heterodimers formed between the Vaccinia-encoded TRα and the endogenous RAR or RXR (Demczuk et al, manuscript in preparation).

All the above experiments were done by adding T3 to unliganded receptors present in nuclear extracts. To verify that receptors saturated in vivo with T3 had similar properties, nuclear extracts were prepared from HeLa cells infected with recombinant Vaccinia virus and grown in excess T3 overnight. Unexpectedly gel retardation analyses (fig 4C) show that the ligand saturation in vivo prevented 2×TR formation on both the Pal 0 and Dir 4 elements. The discrepancies between the in vitro and the in vivo results with Pal 0 were reproducible with three different nuclear extracts from T3 treated cells.

We next determined the effects on DNA binding of different thyroid hormone analogues. Figure 5 shows that 3,3′,5-Triiodothyroacetic acid (Triac), 3,3′,5′-Triiodo-L-thyronine (rT3), L-Thyroxin (T4) and 3,5 Diiodo-L-thyronine (T2) all prevented 2×TR formation on the Dir 4 element, with T2 being the least efficient, requiring 1 μM for full effect. Moreover, the ligands varied in their ability to affect migration of the 1×TR complex: Triac was as efficient as T3 in causing an increased mobility, whereas T2 with the lowest affinity for the receptor was the least potent. Since the concentrations of ligand used were above or close to their Kd values for the receptor, the data suggest that the different ligands have differential capabilities in inducing conformational changes in receptors complexed with DNA.

Half site occupancy by liganded receptors

To determine which of the half sites the ligand-bound receptor occupies on the Dir 4 element, a methylation interference experiment was carried out. For this, unliganded receptor was added to a partially methylated DNA fragment containing the Dir 4 sequence, and then treated with T3. The protein/DNA complexes were separated on a polyacrylamide gel, and the DNA in the −T3 2×TR, −T3 1×TR and the +T3 1×TR complexes were subjected to chemical cleavage. The results (fig 6) demonstrate that the monomeric TR bound preferentially to the 3′ (downstream) AGGTCA half site, irrespective of the presence or absence of ligand, as shown by inhibition of receptor binding by methylation of the 2 G residues in the AGGTCA motif on the upper strand, and of the single G in the TGACCT motif on the lower strand (open circles). Quantification of 5′ and 3′ half site occupancy demonstrated that liganded and unliganded receptors exhibited an identical preference for binding to the 3′ half site (data not shown). The 2×TR complex formed in the absence of ligand, bound to both half sites with equal efficiency (fig 6 lane 4). The data therefore also suggest that both receptors in the 2×TR complex were released from the DNA by T3, and

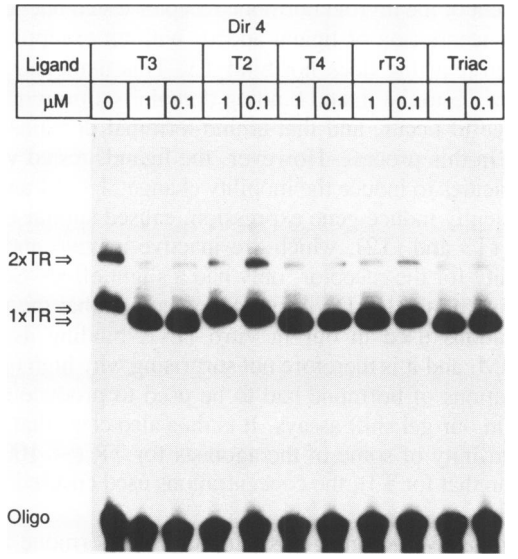

Figure 5. Effects of thyroid hormone analogues on DNA binding. TRα-containing nuclear extracts were incubated with 0, 100 nM or 1000 nM of T3, Triac (3,3',5-Triiodo-Thyroacetic acid), rT3 (3,3',5'-Triiodo-L-thyronine, T4 (L-Thyroxin), and T2 (3,5 Diiodo-L-thyronine). Labelled Dir 4 oligonucleotide was added and complexes were analysed on polyacrylamide gels.

that liganded receptors thereafter rebound to the preferred 3' half site. The preferential binding to the 3' half site is likely to be due to nucleotides present in the spacer sequence between the half sites, since the lower strand G proximal to the 3' half site was under-methylated in the 1×TR complexes (fig 6, open triangle)

Effects of T3 on TR/RAR and TR/RXR heterodimers

At high concentrations, thyroid hormone receptors form homodimeric complexes in vitro on Dir 4 types of elements, binding with low affinity [22]. In contrast, TR/RXR and TR/RAR complexes bind with a much higher affinity [12−18, 22]. To test the effect of ligand on TR heterodimers binding to Dir 4 elements, nuclear extracts containing the respective receptors were mixed, treated with T3, and allowed to bind DNA. Analysis of the types of complexes formed showed that about half of the TR/RAR heterodimers remained bound to the Dir 4 oligonucleotide after treatment with T3 (fig 7A). In contrast, the TR/RXR heterodimers were fully stable in the presence of T3, both on the Dir 4 and Pal 0 elements (fig 7C). It is noteworthy that also the heterodimers exhibited a faster mobility in the gel, indicating that T3 induces a conformational change of the thyroid hormone receptor in complex with other receptor proteins.

Quantification of the TR/RAR complexes (fig. 8, middle panel) showed that total DNA binding was affected by ligand only to a minor degree if at all. However, the reduction in TR/RAR complex seen after addition of T3 was accompanied by a reciprocal increase in the 1×TR band. As a comparison, about 90% of the homodimeric 2×TR complexes were abolished by the hormone (fig 8, left panel), again with the decrease accounted for in the 1×TR fraction. Quantification of the TR/RXR complexes ±T3 confirmed the lack of effect of the hormone (fig 8, right panel). Similar results were obtained with an oligonucleotide representing the response element (Sal) found in the MuLV LTR (data not shown).

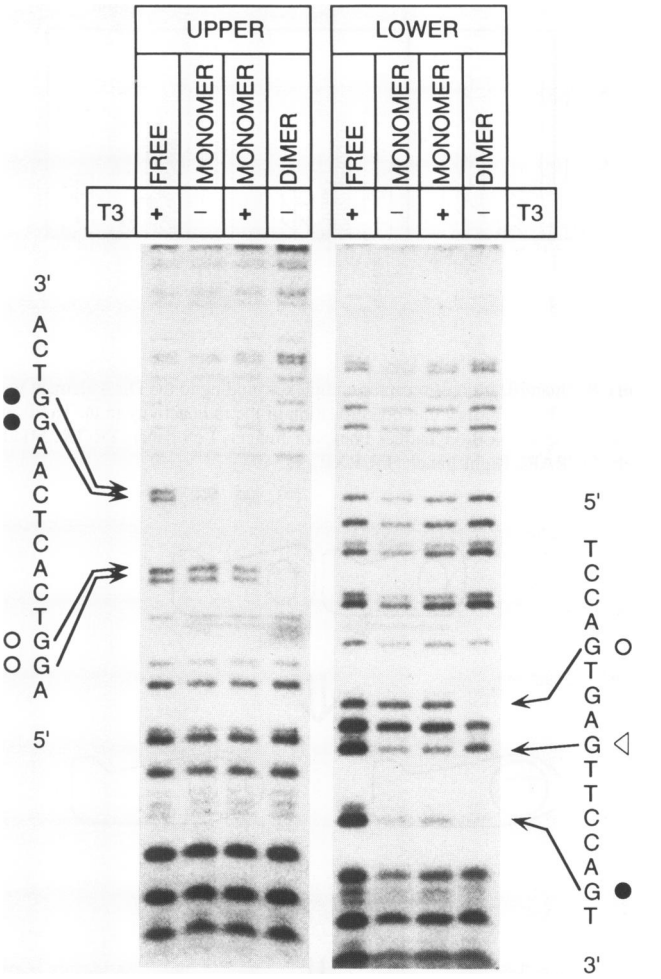

Figure 6. Location of liganded receptor monomer on the Dir 4 element. Partially methylated 114 bp fragment containing Dir 4 in complex with TRα +/− ligand was analysed on denaturing polyacrylamide gel. Labelled upper and lower strand is shown and the Dir 4 nucleotide sequences are aligned for the two strands. Note that the lower strand G proximal to the 3' half site was under-methylated in the 1×TR complexes (open triangle).

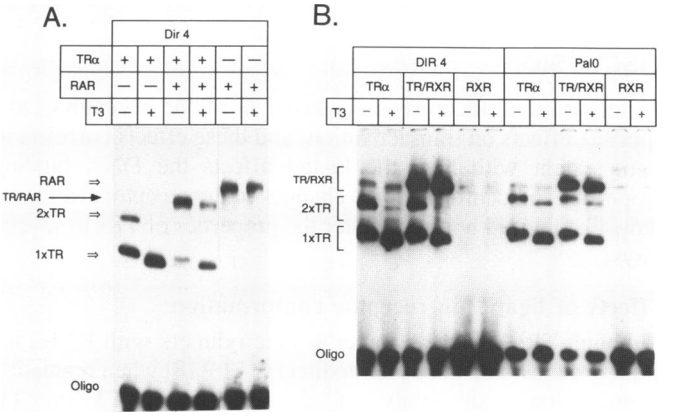

Figure 7. Effect of T3 on receptor heterodimers. **a)** TR/RAR heterodimers on Dir 4 **b)** TR/RXR heterodimers on Dir 4 and Pal 0 .

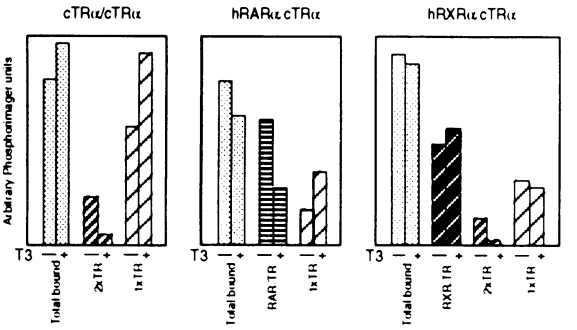

Figure 8. Quantification of receptor complexes binding to the Dir 4 element with and without T3. Arbitrary unit quantification of the radioactivity in the band shift gels was done with a Biodynamics Phosphor Imager. Left panel: TR/TR; Middle panel: TR/RAR; Right panel: TR/RXR.

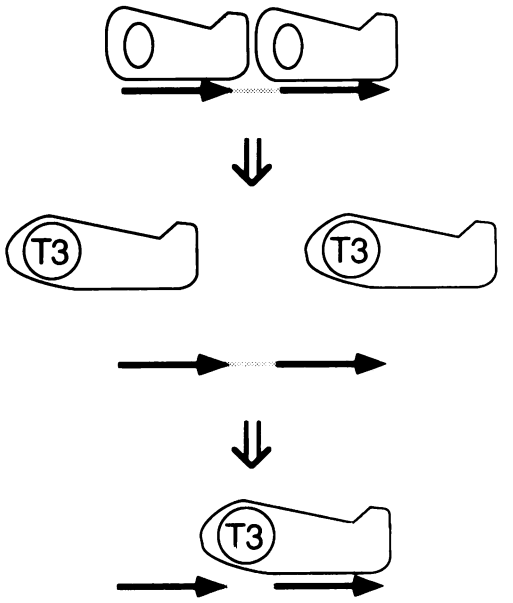

Figure 9. Model of the T3 effects on TRα bound to a direct repeat element.

DISCUSSION

Agonists and antagonists for steroid hormone receptors have opposite effects on transactivation, and these effects correlate to some extent with how the ligand affects the DNA binding properties and conformational changes of the receptor. Here, we show that thyroid hormones alter the properties of TRs in several ways.

Effects of ligand on receptor conformation

Although TRs are likely to act as heterodimers with RXRs (or other proteins) and not as homodimers [12–18] when regulating transcription, our study of the effects of T3 on TR homodimer/DNA complexes has provided important clues to the ligand-induced structural changes in the receptor. Ligands caused all TR containing DNA complexes to migrate faster in polyacrylamide gels, suggesting that they induced a conformational change in the receptor. The change was independent of the thyroid hormone receptor tested but dependent on the concentration of ligand added, with an exception of the v-erbA protein that does not bind T3. This demonstrates that binding of T3 to the ligand binding domain is a prerequisite for the change to occur, and that amino-terminal domains are not involved in this process. However, the ligands tested varied in their capacities to induce the mobility change: T3, T4 and Triac, that efficiently induce gene expression, caused similar changes; whereas rT3 and (T2), which are inactive in vivo and have a low affinity for the receptor, only had a slight effect. Scatchard analyses with labelled DNA as ligand showed that the receptor concentrations used in our in vitro DNA binding assays are 30–60 nM, and it is therefore not surprising why high (100 nM) concentrations of hormone had to be used to produce mobility changes in our gel shift assays. It is thus also clear that, despite the low affinity of some of the agonists for TR (~ 1000 times lower than that for T3), the concentrations used ensured receptor saturation.

Unfortunately, no antagonists for thyroid hormone receptor action are known, and it is therefore not possible to study whether the ligand-induced conformational change correlates with induction of transcription, as has been described for steroid receptors [5, 6]. Nevertheless, the data indicate that TR-DNA binding experiments may be useful for identification of antagonists.

Stability of homodimeric TR/DNA complexes in the presence of ligand

The observation that T3 abrogated TR homodimerization from occurring on the Dir and F2 but not the Pal series of elements suggests that the ligand-induced conformational changes are restricted to a local domain involved in protein contact. In this model (figure 9), the face of the receptors located at the 5′ side of a bound AGGTCA motif in Dir and F2 elements undergoes a conformational change. Consequently, TR homodimers would not be affected when residing on palindromic elements, whereas they, when bound to direct or inverted palindromic repeats, would undergo allosteric changes that do not allow 2 receptor molecules to bind to the adjacent half sites. The fact that methylation of the G residues in the 5′ AGGTCA was found in the T3 treated 1×TR Dir 4 complex furthermore suggests that both receptor molecules were released from the DNA molecule, and that ligand-bound TRs rebound to preferred 3′ half sites in Dir 4 oligonucleotides, methylated in the 5′ AGGTCA motif.

Thyroid hormone receptors can form dimeric complexes on elements with the AGGTCA motif oriented as palindromic, direct, or inverted palindromic repeats, and binding to some of these elements is cooperative [22]. This indicates that different protein interfaces interact to stabilise the 2×TR/DNA complexes, in a manner dependent on the orientation of the receptor proteins. It is thus possible that on a direct repeat, a T3-mediated conformational change causes two effects: i) a lowering of the DNA binding affinity attributable to protein contacts and ii), a steric hindrance, not allowing the juxtaposed receptors to remain bound to DNA. Accordingly, if the distance between the 2 half sites in a direct repeat element is sufficiently increased, a TR/TR complex would not be abolished by ligand, which indeed was the case with the Dir 6 element (Fig 2B). In addition, Scatchard analyses demonstrate that T3-bound TRs have a Kd for DNA twice that of unliganded receptors (data not shown).

The observation that in vitro but not in vivo saturated TRs formed dimeric complexes on the Pal 0 element remains

unexplained. TRs have a long half life in vivo (3–6 h; [38, 39], and it is possible that downregulation of the T3 signal is mediated in vivo by a modification of the receptor protein affecting its DNA binding properties. However, we have failed to detect major alterations in in vivo saturated receptors using SDS-PAGE analysis (data not shown).

Heterodimeric receptor/DNA complexes

Our data showed that ~50% of the RAR/TR but none of the RXR/TR DNA complexes were dissociated by T3. Several reasons for why RXR/TR heterodimers are stable in the presence of T3 can be postulated. It is possible that the heterodimer is polar when binding to a direct repeat, i.e. that the TR occupies the upstream and the RXR the second repeat or vice versa. If the TR occupies the first position, a T3 induced conformational change could take place in a domain located distal to the RXR, and would therefore not affect the heterodimer. Alternatively, the RXR could reside on the first and TR on the second AGGTCA motif. In such a situation the TR would undergo a conformational change upon binding of T3 while leaving dimerization interfaces unaffected, and might therefore not affect the DNA binding properties of the heterodimer. Our present data do not allow a discrimination between these two alternatives, although they argue against a third possibility: the RXR/TR heterodimer, in contrast to the TR/TR homodimer, has a low kd (1.4 nM vs. 17 nM; [22]) for the Dir 4 element, and it could be argued that this renders the former complex resistant to T3. We consider this unlikely, since TRs form homodimers on the inverted palindrome F2 with a kd similar to that found for RXR/TRs on the Dir 4 element [9].

The partial stability of the RAR/TR complex could be due to several reasons. In contrast to the situation with RXRs, RARs have no synergistic effect on TR mediated transactivation from Dir 4 type of elements [40]. It has thus been proposed that RARs, unlike RXRs, do not transactivate by heterodimerization with TRs [13–18, 41], although repression by TRs of RAR responsive genes through RAR/TR heterodimerization (as opposed to outtitration of available RXRs by TRs) has not been ruled out. Nevertheless, it is possible that RAR/TR complexes represent in vitro artefacts, and that the TR molecules can occupy either AGGTCA motif. Accordingly, a liganded TR in the second position could dislodge the receptors from the DNA, whereas the protein/DNA complex would be stable when the receptors are present in the reverse order.

While this manuscript was in preparation, Yen et al [42] reported that thyroid hormones only allow monomeric TR binding to the F2 element and a natural element resembling the Dir 4 oligonucleotide. Our results considerably extend these data. For instance, we show that TR homodimer formation on palindromic elements is resistant to hormone in vitro, T3 acts before and after DNA binding has occurred and is dependent on the distance between the half sites in direct repeats, the effects of ligand can be reproduced with receptors saturated in vivo with T3, and protein/DNA complexes can be visualised with specific antibodies or radiolabelled ligand.

ACKNOWLEDGEMENTS

We thank Mrs. Gunnel Jönsson for secretarial help and Dr Örjan Wrange for critically reading the manuscript. We are grateful to Dr H. Stunnenberg for the gift of Vaccinia virus strains. M. H. was supported by a grant from the Deutsche Forschungsgemeinshaft. This project was funded by grants from the Swedish Cancer Society (RMC), the Natural Science Foundation (NFR), The Swedish Technical board for Development (STUF), The Beijer Foundation, Göran Gustavsson's Foundation, Svenska Sällskapet for Medical Research and funds at the Karolinska Institute.

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