Transactivation by the thyroid hormone receptor is dependent on the spacer sequence in hormone response elements containing directly repeated half-sites

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Received March 19, 1996; Revised and Accepted May 1, 1996

ABSTRACT

The thyroid hormone receptor (TR) regulates the transcription of its target genes by interacting with specific hormone response elements consisting usually of directly repeated half-sites with the consensus sequence AGGTCA. To investigate the role of the spacer sequences separating the half-sites, heterodimers formed by TRα and the retinoid-X receptor (RXR) were used in a PCR based selection and amplification assay. The TRα/RXR heterodimer selected for elements with directly repeated half-sites having a spacer of 4 nucleotides (DR4). Preferences for nucleotides in the TR binding half-site motif as well as for the 4 nucleotides separating the two half-sites were found. DNA binding and transfection studies using DR4 elements with different spacer sequences showed the importance of these nucleotides for the activity of the response element: some spacer sequences allowed little or no transactivation from the element, whereas other sequences supported strong transactivation. A pyrimidine nucleotide in position three of the spacer enhanced TRα binding and transactivation. Additional experiments showed that heterodimers between RXR and other putative receptors exhibited a similar but distinct specificity for the spacer sequence. Our results thus suggest that the four nucleotides separating the two half-sites in hormone response elements have a major role in determining induction of hormone responsive genes.

INTRODUCTION

In vertebrate organisms a multitude of cellular events such as growth, development, differentiation and homeostasis are regulated by thyroid hormones, which affect their target cells by interacting with intracellular thyroid hormone receptors (TRs) (1). The thyroid hormone receptor was identified as a ligand-dependent transcription factor which belongs to the steroid/thyroid hormone receptor superfamily of nuclear receptors (1–3). The members of this gene family share high structural similarities, particularly in their DNA binding domains (4–6). Due to their DNA binding domains, these receptors interact with specific hormone response elements in their target genes. These elements contain two different core recognition motifs (half-sites) which were previously identified for the glucocorticoid (AGAACA) (7) and the estrogen receptor (AGGTCA), respectively (8). Since the recognition motifs for all members of the nuclear receptor family are highly conserved, the specificity of hormone mediated signal transduction is regulated by the arrangement of these recognition motifs in dimeric or multimeric receptor-specific binding sites (9).

Thyroid hormone response elements (TREs) contain two half-sites of the AGGTCA motif (9), which can be arranged as direct repeats (DR), inverted repeats (IR) or everted repeats (ER) (10). TR binds to and transactivates from these elements as a heterodimer together with the retinoid-X receptor (RXR) (11–15). RXR heterodimerises not only with TR, but serves as the partner for various nuclear receptors including those for retinoic acid, vitamin D3, and a number of orphan receptors for which no ligands have yet been identified (16). For response elements of the direct repeat type, the number of nucleotides separating the two half-sites specifies receptor specificity (17,18). Accordingly, TR–RXR heterodimers bind preferentially to elements spaced by 4 nucleotides (nt) (DR4). In these complexes RXR occupies the 5′ half-site while TR, located at the 3′ half-site of the element, determines the specificity (19–21). RXR augments thyroid hormone mediated transactivation by increasing the affinity of TRs for binding to the cognate response elements.

The analysis of promoters from TR target genes led to the identification of natural TREs, of which most elements are of the DR4 type (18,22–26). However, none of these elements contains two copies of the consensus AGGTCA motif. In addition, in some elements, like in the rat growth hormone promoter, more than two half-sites were found which are arranged as a direct and an inverted repeat (27–30). The principles by which these complex elements govern transcription of target genes are not yet fully understood. The variations in the sequence and structure of response elements and the possibility of TR isoforms to...
heterodimerise with different forms of RXRs offer flexibility in modulating ligand-dependent transactivation. To characterise the structural requirements within the spacer sequence of a TRE, we applied a PCR-based approach to select for an optimal binding site for TRα–RXR heterodimers. The identified sequences shows that TRα in this assay preferentially binds to direct repeats separated by 4 nt, and that the nucleotide composition of the sequence separating the repeats influences DNA binding affinity as well as transactivation properties of TRs. Our data also suggest that other, still unidentified proteins, have similar but distinct requirements on the composition of the spacer.

**MATERIALS AND METHODS**

**Preparation of nuclear receptors**

cTRα and mRXRγ were expressed as histidine-tagged fusion proteins in a Vaccinia virus expression system. The histagged cTRα was described earlier (31). A mRXRγ cDNA was cloned into the EcoRI site of pATA-18-His (32) and used for recombinination into the Vaccinia virus genome as described (33). Nuclear extracts from infected HeLa cells were prepared as described by Vivanco Ruiz et al. (34). The histagged receptors were purified from nuclear extracts on a Ni2+-NTA matrix (Diagen, Hilden, Germany) as described by Janknecht et al. (35). After chromatography, the receptors were concentrated by ammonium sulphate precipitation [0.295 g (NH4)2SO4/ml], dialysised against a buffer containing 20 mM HEPES (pH 7.9), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT, and stored at –70°C. For preparation of His-cTRα–His-mRXRγ heterodimers HeLa cells were co-infected with viruses encoding His-cTRα and His-mRXRγ. The heterodimers were extracted from these cells and purified as described for the other receptors. These extracts contained almost exclusively heterodimerised His-cTRα and His-mRXRγ (data not shown). The expression of unmodified receptors (cTRα and mRXRα) was described in reference (32).

For the preparation of whole cell extracts from JEG cells, cells from confluent 10 cm dishes were washed with PBS, scraped off the plate and collected by centrifugation. The pellet was then extracted on ice with 150 µl of a buffer containing 50 mM Tris–HCl (pH 7.5), 440 mM NaCl, 5 mM MgCl₂, 0.5% Triton X-100, 10 mM DTT, 0.2 mM PMSF and 200 U/ml trasylool (Bayer, Leverkusen, Germany). After 30 min the extracts were cleared by centrifugation and stored at –70°C.

**Selection and amplification of high-affinity binding sites**

Gel retardation assays were performed and quantified as described by (36)—for supershifts 1 µl antisumern was added to the reaction mixture and then incubated for further 30 min on ice before loading the gel. The anti–TRβ antisumern was described by (37); the anti–RXR antisumern was a gift from P. Chambon.

For the selection of an optimal binding site the following oligonucleotides were synthesised (SGS, Ksping, Sweden):

- **Template:**  
  5′-CCC GAA TTC CCC ACT GCT TAT AGG TCA N3′  
  TAA GCT TCA GGG AGG CGA CAG-3′

- **Upper primer:**  
  5′-CCC GAA TTC CCC ACT GCT TAT-3′

- **Lower primer:**  
  5′-CTG TCG CCT CCC TGA AGC TTA-3′

The primers were labelled at their 5'-ends using [γ-32P]ATP (Amersham, Buckinghamshire, UK) and T4 polynucleotide kinase (Promega, Madison, USA) and then applied to PCR. In the first reaction, 5 pmol template were amplified with 30 pmol of each labelled primer during 20 cycles of 1 min at 94°C, 1 min at 62°C and 1 min at 72°C (Taq polymerase and 10× buffer were obtained from Promega, Madison, USA). The PCR product was purified in a 10% non-denaturing polyacrylamide gel and extracted with TE buffer. The labelled PCR product was then incubated with purified His-cTRα–His-mRXRγ heterodimers. Bands co-migrating with His-cTRα–His-mRXRγ bound to a DR4 element were excised, and the DNA extracted with TE buffer. The DNA was used as template in a second PCR reaction, for which again 30 pmol of each labelled primer was used (25 cycles of 15 s at 94°C, 15 s at 62°C and 15 s at 72°C). After four rounds of selection and amplification a strong gel shift with the PCR product was observed. DNA from this complex was amplified and cloned into the EcoRI and HindIII sites of pUC19. Positive clones from the colour selection were picked and used for preparation of plasmid DNA. DNA sequence analysis was performed with a M13 universal primer (New England Biolabs, Beverly, USA) and a Pharmacia sequencing kit (Sollentuna, Sweden).

**Oligonucleotides and DNA constructs**

Synthetic oligonucleotides encoding the different DR4 elements were obtained from SGS (Ksping, Sweden) and cloned into the HindIII site of the reporter gene vector pBlCat (38). The double-stranded oligonucleotides had the following sequences:

- For an extended half-site:  
  5′′ agcttcAGGTCAagcttca 3′′  
  3′′ agTCCAGTtgagaagttcga 5′′

- For AGG TCA atca AGG TC:  
  5′′ agcttcAGGTCAatcaAGGTCCa 3′′  
  3′′ agTCCAGTttgagttcga 5′′

- For AA AGG TCA AAAGG AGG TCA:  
  5′′ agcttaaaAGGTCAaaaaAGGTCA 3′′  
  3′′ attTCCAGTttttTCCAGTtcga 5′′

- For other DR4 elements:  
  5′′ agcttcAGGTCAaaaaAGGTCA 3′′  
  3′′ agTCCAGTnnntnTCCAGTtcga 5′′

The sequences of the spacer regions (nnn) are indicated in the text and figures. The clone for cTRα in the expression vector pSG5 was described in reference (36), VP16–V3 and VP16–V3–DBD were described in reference (32), mRXRα and γ in pSG5 were gifts from P. Chambon, VP16–hRXRα and VP16–hTRβ were gifts from T. Perlmann.

**Cell culture, transfections and CAT assays**

JEG cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 8% fetal calf serum and antibiotics. For transfection 2 × 10⁵ cells were seeded onto 35 mm dishes and transfected the next day by a calcium phosphate procedure (25) with 100 ng expression vector, 500 ng reporter gene vector and 250 ng of a RSV-β-galactosidase control plasmid. The medium was replaced with T3 depleted medium, or medium containing 25 nM 3,3′,5-triiodothyronine (T3) 12 h after addition of precipitated DNA. For transfection with the VP16-constrasts only T3 depleted medium was used. Cells were harvested after 24 h and the CAT as well as the β-galactosidase assays were done using standard protocols (39). The induction rates were normalised for the transfection efficiency by using the β-galactosidase activities (40).
all transfections employed duplicate samples, and were done at least twice. Representative experiments are shown in the figures.

RESULTS

Selection for an optimal binding site for TRα–RXR

To characterise an optimal binding site for TRα, we applied a PCR-based selection and amplification approach which would allow selection of an optimal 3′ half-site and a spacer sequence (41). An oligonucleotide was synthesised in which one half-site of the AGGTCA motif was present in the 5′-position followed by 12 random base pairs (bp). Since RXR has been shown to interact exclusively with the 5′-half-site of response elements bound by TRα–RXR heterodimers, this template was expected to select for a TRα specific binding site in the 3′-position. cTRα and mRXRγ used in the selection were expressed as histidine-tagged fusion proteins in a Vaccinia virus expression system and purified as heterodimers by affinity chromatography.

The heterodimers formed an easily detectable complex with the amplified PCR product after four rounds of selection that co-migrated in a gel retardation assay with a heterodimer complex bound to a DR4 element. The resulting PCR products were cloned into pUC19 and sequenced. The sequences from 12 individual clones established a DR4-like binding site containing the consensus sequence atcaAGGTCC 3′ to the fixed AGGTCA motif. This sequence differed in the last position from the AGGTCA half-site previously identified as a core recognition motif. This sequence differed in the last position from the consensus sequence atcaAGGTCC 3′. The synthetic elements bound not only to TRα–RXR heterodimers but also to TRα homodimers and monomers. However, differences in binding to receptor complexes were found: the element with the CTTC spacer bound TRα heterodimers substantially better than the TRα homodimers and monomers. However, differences in binding to receptor complexes were found: the element with the CTTC spacer bound TRα heterodimers substantially better than the other elements, whereas monomer binding was equal. Since the three synthetic elements differed only in the spacer sequences we conclude that the nucleotides separating the half-sites modulate the binding of two receptor molecules to a DR4 type element.

The sequence of the spacer modulates transactivation from DR4 elements

The DNA binding data described above showed that DR4 elements with different spacer sequences had distinct affinities for TRα. To determine the importance of the sequence of the spacer and to show the influence of the adenine/cytidine exchange in the last position of the second half site, oligonucleotides representing the different elements were cloned into a reporter plasmid and tested in transactivation experiments in JEG cells. The results (Fig. 3A) show that the cytidine in the last position strongly reduced hormone induction as compared to the element with an adenosine. The data also show that the element with the sequence AGGTCA CTTC AGGTCA yielded a >2-fold higher induction as compared to a similar element containing an

Figure 1. Binding of TR to the selected DR4 element AGGTCAatcaAGGTCC. Synthetic DR4 elements containing the AGGTCAatcaAGGTCA and AGGTCAatcaAGGTCC sequences were compared for binding to Vaccinia virus expressed TRα in a gel retardation assay. The elements are indicated by the sequences of their second half-sites, shifted complexes contained TR monomers (TR) and homodimers (TR/TR).

Figure 2. Binding of receptors to different DR4 elements with AGGTCA half-sites. Synthetic DR4 elements with the spacer sequences CTTC, CTCA and CCAT were incubated with Vaccinia virus expressed cTRα (lanes 1), mRXRα (lanes 2) and cTRα plus mRXRα (lanes 3). Shifted complexes containing TR monomers (TR), TR homodimers (TR/TR) or TR–RXR heterodimers (TR/RXR) are indicated. The Vaccinia virus extract for mRXRα showed some unspecific binding which was also found with extracts from HeLa cells infected with wild-type viruses (data not shown).
ATCA spacer, whereas the spacer sequence CCAT, representing the ‘anti-consensus’ of the PCR selection, was inactive.

To determine further the importance of the individual nucleotides in the spacer, a set of elements was constructed in which adenosines were replaced, one by one, in one of the nucleotides in the CTTC sequence. The subsequent transactivation experiments showed (Fig. 3A, middle section) that replacement of any of the nucleotides reduced activity. However, changes in positions 1 and 3 led to the strongest reductions in hormone induced transactivation.

The influence of a purine or a pyrimidine in the first position of the spacer was tested by comparing DR4 elements which had an adenosine or a cytidine in this position, combined with additional changes in positions 3 and 4 of the spacer. For elements with a cytidine in the first position and a thymidine in the third position gave a significantly higher transactivation than those with a cytidine in the first position and an adenosine in the third position. In contrast, for the elements with an adenosine in the first position and the CTCA spacer element, the transactivation was highest.

The transactivation experiments in JEG cells were repeated by cotransfecting TRα and RXRα to examine the influence of increased levels of RXR on the constraints conferred by the spacer sequences used above. Figure 3B shows that this reduced the influence of the spacer sequence, particularly in case of the weakly active spacers (ATTA, ATAC, CTCA, CTAC). It is noteworthy, though, that the ATTC and the ‘anti-consensus’ CCAT sequences remained inefficient or inactive. Figure 3B also shows that co-expression of RXR did not alleviate the low inducibility from an element with a cytidine in the last position of the second half-site. The data in Figure 3 suggest that ≥ 2 nt in the spacer sequence determine in concert the ability of TRα to transactivate from DR4 type of elements, and that highly expressed RXR in many instances but not all can render otherwise inefficient elements more active. Careful titration of the amount of RXR expressing plasmid, to be used in these experiments, has been done to exclude squelching phenomena (data not shown).

**Transactivation by TRα correlates with its binding to the response elements**

The cotransfection experiments with JEG cells demonstrated the importance of the nucleotides in the spacer region in DR4 type of elements. To examine whether the reduced transactivation correlated with receptor binding to these elements, gel retardation assays were done. Vaccinia virus expressed TRα was incubated with the elements with low activity (spaced by CTCA and CCAT), or with the element spaced by CTTC and its derivatives. The result (Fig. 4A) shows that elements with a reduced activity in the transfection studies had also a reduced ability to bind to the receptor: nearly no TRα homodimer binding was detected to elements that gave background levels of transactivation. Parallel gel retardation experiments (data not shown) with TRα–RXR heterodimers showed no or little difference in binding.

To compare the in vitro DNA binding affinity with that in vivo, we constructed a receptor that transactivates in the absence of ligand in transfected JEG cells. For this, we added the transactivating domain from the viral protein VP16 to a gag–TRα fusion protein, yielding the VP16–V3 construct (32). The transactivation observed with this construct in the absence of ligand reflects DNA binding affinity (32). Figure 4B shows that this receptor chimera exhibits a spacer-dependent transactivation. In most instances the elements that gave the highest hormone dependent activation also yielded the highest VP16-mediated transactivation. The differences between the individual elements were enhanced in the assay: the element with the spacing sequence CTTC gave a 50-fold activation, whereas the ‘anti-consensus’ spacer CCAT gave only a 2-fold activation. Moreover, JEG cells transfected with VP16–V3 plus RXRα gave a similar pattern of transactivation as that obtained without RXR (data not shown). The reporter constructs used in this assay differ only in the 4 nt separating the AGGTCA half-sites. The results therefore suggest...
Figure 4. Correlation between cTRα binding and transactivation. Different DR4 elements were studied for their binding to Vaccinia virus expressed cTRα as monomers (TR) and homodimers (TR/TR) in a gel retardation assay (A). The elements are named by the sequences of their spacers. (B) The transactivation from different DR4 elements by the VP16-activated chimeric receptor VP16–V3 in transfected JEG cells. The elements used are indicated as described for (A).

that the spacer region determines the binding of TRα to response elements of the DR4 type also in vivo.

The spacer sequence also determines transactivation by other receptors

Next we investigated if the spacer sequence in DR4 elements also determine transactivation by other receptors. Several orphan receptors have been shown to heterodimerize with RXR or differently spaced response elements of the direct repeat type (42–44, 58). We therefore tested the transactivation by VP16–RXR chimera from the DR4 elements with AGGTCA half-sites. For this, we compared the VP16-mediated transactivation by the VP16–V3 and the VP16–RXR constructs in JEG cells. The experiments show (Fig. 5) that VP16–RXR exhibited a similar but yet distinct selectivity for the spacer sequence as compared to the control VP16–V3 construct. The AGTG spacer gave the strongest activation with VP16–RXR, VP16–V3 was the most active with the CTTC sequence, whereas the ATTC and CTCA spacer gave poor activation for both receptor constructs. In addition, the ‘anti-consensus’ CCAT gave an appreciable activation with VP16–RXR and was inactive with VP16–V3.

To verify that the VP16–RXR chimera heterodimerized with a receptor(s) distinct from TR, nuclear extracts from JEG cells were used in a gel retardation assay with the CTTC DR4 element. The results (Fig. 6) show that a complex formed by cTRα plus mRXRα and factors from JEG cells were analysed for their receptor content with anti-TR (lanes 2 and 6) and anti-RXR-specific antibodies (lanes 3 and 7).

We conclude that a spacer sequence in a DR4 element can modulate the DNA binding properties of complexes consisting of...
a heterodimer between RXR and an unknown transcription factor, possibly a receptor like protein.

**DISCUSSION**

**The PCR selected recognition motif for TRα**

To understand better the structure and function of TRES, we applied a PCR-based selection and amplification approach (41) for identification of an optimal TRα–RXR binding site. TRα–RXR selected a direct repeat separated by 4 nt with an AGGTCC consensus binding site for TRα. This confirmed the earlier results from a similar selection approach (21), that identified a spacer length of 4 nt as specific for TRα. The AGGTCC sequence found in our selection has also been found in some natural elements (18,25) and it is very similar to the AGGTCAT motif, which is often found in natural response elements that confer high induction by TR and related nuclear receptors. We also observed a high selectivity for a cytidine instead of an adenosine in the last position of the half-site motif (10 out of 12 clones). DNA binding and cotransfection experiments with DR4 elements only distinct by an adenosine or a cytidine in this position showed a clear reduction in binding and transactivation due to the cytidine.

Obviously, the cytidine in the tested DR4 element (AGGTCA ATCA AGGTCC) destabilised DNA binding and by that also reduced the transactivation from this element. The natural TRE from the MoMLV also contains a cytidine in that position (GGGTCA TTTC AGGTCC) (59). Transfection experiments with the VP16-activated chimera VP16–V3 showed for this TRE a 4-fold reduced binding as compared to the similar element CTTC (AGGTCA CTTC AGGTCA). These data suggest that a cytidine in the last position of the second half-site is not optimal for TR binding, although, a TRE with an optimal spacer sequence and an optimal first half-site can be functional as was shown for the MoMLV element. In fact, a previous report demonstrated receptor interaction with an adenosine in the last position of the 3’ half-site (45). We conclude that since the cytidine in the last position of the recognition motif clearly neither favours DNA-binding, nor transactivation, its selection is likely to reflect constraints inherent in the PCR-based selection approach.

**TRα–RXR preferred certain nucleotides in the spacer region of DR4 elements**

Using the PCR based selection we detected not only preferences for certain nucleotides in the second recognition motif but also in the 4 bp separating the two half-sites. To study further the importance of the specific nucleotides in the spacer sequence for the binding and transactivation from DR4-like response elements, we changed each nucleotide one by one to an adenosine in the synthetic CTTC spacer sequence, and tested the activity of the elements in both DNA binding and transactivation experiments. Both the PCR selection and the DNA binding experiments showed that adenosines were preferred in position one of the spacer. However, the high binding affinity was contrasted by a poor transactivation in JEG cells. The second and the fourth positions in the spacer were of less importance, since changes of a thymidine or cytidine into an adenosine did not change, or had only minor effects, on the activity of these elements in any of the assays used. The results demonstrated, however, a strong dependence on pyrimidines in position 3. Only pyrimidine nucleotides were found in position 3 during the PCR selection (with the exception of one clone), indicating that purines in this position destabilize TRα binding. Similar conclusions were reached from gel shift experiments in which a thymidine to adenosine exchange in position 3 dramatically reduced TR binding in vitro. Furthermore, transactivation studies using elements with an adenosine in position three (CCAT, CTAC and ATAC) showed very low activity, thus supporting our suggestions.

The data from our different binding and transactivation experiments indicate that TRα binds probably to an extended half-site motif which overlaps with the spacer region. In previous publications other groups reported that TR binds as a monomer to a 8 bp binding site (46,47). According to these data, a 5’-flanking TA (46) or TG (47) motif stabilises binding so that these elements can be activated by TR monomers. In our cotransfection experiments that compare the activity of DR4 elements spaced by CTTC, CTG or CTTA, the CTTC and CTG elements showed a similar activity while CTTA had a reduced activity (data not shown). The experiments, however, gave no evidence for a significant transactivation from a half-site with a CTTC 5'-flanking sequence (data not shown).

Despite our testing of a very large number of elements with different spacer sequences (Figs 3 and 6, and data not shown), no simple rule could be established for the preferred nucleotides. For instance, the sequence ATTC gave lower transactivation as compared to CTTC, whereas the sequence ATCA was more efficient than CTCA. This indicates that the structure or overall composition is of importance, possibly for allowing proper binding of the element. Alternatively, it is possible that some spacer sequences together with their flanking sequences form a recognition motif that binds other, interfering transcription or chromatin associated factors in vivo. Such a mechanism may be responsible for the low transactivation from the element with the selected ATCA spacer.

Several reports on orphan receptors have demonstrated that nucleotides upstream of the binding site greatly influence receptor binding. Wilson et al. (48,49) showed that the ‘A-box’ domain of NGFI-B, located in between the DNA and ligand binding regions, conferred specific interaction with adeninoses upstream of the AGGTCA half-site. For these reasons we replaced the A-box region of TRα with the corresponding domain of NGFI-B (data not shown). The chimeric receptor showed increased transactivation form DR4 elements with a preference for adeninosine rich spacers like NGFI-B, however, it was still able to transactivate efficiently from an element with a CTTC spacer (data not shown). It is therefore likely that the specificity of TRs for the spacer sequence is conferred not only by the A-box region. Other experiments characterising NGFI-B demonstrated that amino acids C-terminal to the A-box (the ‘T-box’) supports the A-box in contacting DNA (49). However, a second chimeric TRα with both the T and A-box regions from NGFI-B completely failed to transactivate (data not shown). Mutations in the T-box of NGFI-B and TR have been shown to inhibit or reduce DNA binding, which may explain the inactivity of our chimeric receptor (49,21).

Recent reports have suggested that also the N-terminal domains of nuclear receptors can modulate DNA binding. Mutations in the N-terminal region of the oncogenic form of TRα, v-erbA, have been shown to reduce the binding to response elements with a AGG ACA half-site (50,51). Likewise, in the ROR orphan receptor the N-terminal domains of the α1 and α2 isoforms confer DNA binding specificity for the nucleotides 5’ to the
consensus monomer response element (52). We consider it unlikely that the N-terminal domain of TRα confers the specificity for the spacer sequence, since a VP16 activated hTRβ, VP16–V3, and the normal cTRα exhibited the same transactivating specificity from different DR4 elements despite their distinct N-terminal domains (data not shown).

**Influence of RXR on TRα-mediated transactivation from DR4 elements**

Our experiments demonstrated that the sequence separating the two half-sites in a directly repeated response element influences the ability of thyroid hormone receptors to bind to and to transactivate a target gene. However, the influence of the spacer sequence was diminished by RXR both in the DNA binding and the transactivation experiments. The transactivation directly correlated with the DNA binding ability in *vitro* of the involved receptor proteins, suggesting that the spacer sequence also in *vivo* determines receptor binding. Our data also indicate that limiting concentrations of TRα–RXR heterodimers in a cell, such as in JEG cells transfected with only a TRα expressing plasmid, increases the selectivity for thyroid hormone response by allowing receptor binding preferentially to high affinity response elements. In the RXR co-transfected cells the heterodimer concentration would be higher, allowing receptor heterodimers to bind also to elements with low affinity. Our results with JEG cells were confirmed by experiments using HeLa cells with the VP16-activated TRα fusion protein VP16–V3: the VP16-mediated transactivation from the different DR4 elements showed a similar pattern as observed in JEG cells, but the differences between the elements were less pronounced in the HeLa cells (data not shown). It is possible that the different results obtained with the two cell lines are due to their distinct amounts of RXR (13,53).

The binding to an extended binding site in some DR4 elements can support especially the binding by TRα homodimers. Our *in vitro* binding studies and the transfection experiments with TRα showed good correlation between DNA binding in *vitro* and ligand-dependent transactivation in TRα transfected JEG cells. In these experiments high transactivation from the CTTC element was already maintained in none RXR transfected cells and cotransfection of additional RXR did not increase the transactivation from this element (Fig. 3). Since JEG cells contain endogenous RXR, however, we could not demonstrate that transactivation from this element involved TRα homodimers in *vivo*. In addition, we and others have shown earlier, that TRα homodimers are not stable under ligand treatment on DR4 elements (54–56). The disruption of the homodimeric complexes was also observed in gel retardation assays using a strong TRα homodimer binding element like CTTC (data not shown). The instability of TRα homodimers in the presence of ligand, however, does not exclude a biological function of TRα homodimers interacting with strong binding sites in *vivo*. Under RXR limiting conditions, TRα homodimers could function as repressors on such response elements, which are relieved from their response elements in presence of ligand. Such a mechanism was demonstrated for TR mediated repression and activation on an IR0 element in an *in vitro* transcription system (57).

Some elements, for instance the one with the spacer CCAT, were unable to allow TR-mediated transactivation despite appreciable TRα or TRα–RXR binding in *vitro*. This indicates that even in the presence of RXR some DR4 elements cannot function as response elements for TRα. Indeed, our experiments showed that high transactivation with VP16–RXR chimeric protein via DR4 elements with the CCAT spacer, suggests that other orphan receptors heterodimerize with RXR on this type of elements. While orphan receptors like rev-erbα and ROR bind to their response elements as monomers, two recently described orphan receptors were shown to bind as heterodimers with RXR to DR4 elements (42,44,58). However, additional experiments have shown that the factors in the JEG cells that heterodimerize with RXR are distinct from the LXR and OR orphan receptors (32). Taken together, the data suggest that receptors other than TR that heterodimerize with RXR on DR4 elements, exhibit a preference for the spacer sequence that is similar to but distinct from that of TR.

**ACKNOWLEDGEMENTS**

We are grateful to Dr Pierre Chambron for anti-RXR antibodies as well as cDNAs encoding mouse RXRα and γ, and to Dr Thomas Perlmann for the gift of expression vectors for VP16–hRXRα and VP16–hTRβ. We would also like to thank Drs Thomas Perlmann and Marco Tini for critically reading the manuscript, and Dr Martin Privalsky for sharing data prior to publication. MH was supported by a grant from the Deutsche Forschungsgemeinschaft; GW was supported from the Swedish Society for Medical Research. This project was funded by the Swedish Cancer Society, The Beijer Foundation, Gran Gustafsson’s Foundation, The Swedish Society for Medical Research, Lars Hierta Foundation and funds at the Karolinska Institute.

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