Functional Characterization of a Mutant Thyroid Hormone Receptor in *Xenopus laevis*

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Thyroid hormone plays a causative role during frog metamorphosis, and its effect is mediated by thyroid hormone receptors (TRs). To investigate the function of *Xenopus* TRs, we have recently developed a thyroid hormone dependent *in vivo* transcription system by introducing TRs and RXRs (9-cis-retinoic acid receptors) into *Xenopus* oocytes. Interestingly, using this system, we have found that the TRaβ cloned previously is defective in transcriptional activation compared with TRaA. In *vitro* DNA binding experiments show that TRaB-RXR heterodimers have drastically reduced affinity for a thyroid hormone response element. Site-directed mutagenesis shows that two of the seven amino acid residues that differ between TRaA and TRaB are responsible for the defect in TRaB function. These two residues affect the DNA binding by both TR-RXR heterodimers and TR homodimers. In contrast, heterodimer formation with RXRs is not affected as demonstrated by communoprecipitation and dominant-transcriptional inhibition experiments. By cDNA and genomic DNA sequence analysis, we have demonstrated that the residues, which affect TRaB function when mutated, are identical between the wild type TRaB and TRaA. Thus, our experiments have discovered the first amphibian TR mutant. The DNA binding and transcription activation functions of the mutant are discussed in relation to the recently published TR crystal structure.

Thyroid hormone receptors (TRs) belong to the superfamily of steroid hormone receptors (1–4), which also include receptors for 9-cis-retinoic acid (RXRs), and glucocorticoid receptor, etc. Members of this family are transcription factors whose activity is regulated by their cognate hormones. They have similar structural domains. The DNA binding domain, located in the NH2-terminal half of the receptor, shares considerable homology among different receptors and recognize specific hormone response elements, e.g. TREs (thyroid hormone response elements) for thyroid hormone receptors. The hormone binding domain, on the other hand, is unique for each hormone receptor and is located in the carboxyl half of the protein. Similarly, the other domains are very divergent in sequence among different receptors.

While TR can bind to TREs weakly as homodimers and monomers, it binds TREs with much higher affinity as heterodimers with some other members of the superfamily, especially RXRs (5–9). Furthermore, TR-RXR heterodimers can mediate specific gene regulation by thyroid hormone in tissue culture cells. In addition, RXRs are expressed in most, if not all, tissues. These results suggest that TR-RXR heterodimers are likely to be the active complexes *in vivo* to mediate the effects of thyroid hormone.

We are studying the regulation and function of TRs during amphibian metamorphosis. This postembryonic process is entirely controlled by thyroid hormone and systematically transforms different tissues of a tadpole (10, 11). Thus, blocking the synthesis of endogenous thyroid hormone inhibits the process, whereas the addition of thyroid hormone to the rearing water of premetamorphic tadpoles induces precocious metamorphosis. Furthermore, the response to thyroid hormone is organ autonomous (10–13), suggesting that thyroid hormone acts on individual organs directly through its receptors. Indeed, four TR (TRαA, TRαB, TRβA, and TRβB) genes have been cloned in *Xenopus laevis* (14, 15) and found to be expressed in all metamorphosing tissues of a tadpole (16–21). More importantly, by analyzing the mRNA levels for both TR and RXR genes in different organs during development, we have observed a coordinated regulation of TR and RXR genes (21). Both TR and RXR genes are temporally regulated in an organ-specific manner. High levels of their mRNAs are present in a given organ when it undergoes metamorphosis, while low levels are generally observed in the same organ before or after metamorphosis. These results provide strong evidence that TR-RXR heterodimers mediate the causative effects of thyroid hormone during metamorphosis.

To study the function of these amphibian TRs, we have recently developed an *in vivo* thyroid hormone-responsive transcription system (21, 22). This is achieved by taking advantage of the fact that *Xenopus* oocytes have only very low levels of TRs and RXRs that are incapable of activating a TRE-containing promoter (20–22). In addition, it is easy to introduce exogenous receptors by injecting their mRNAs into the oocyte cytoplasm. Thus, when a single-stranded reporter plasmid containing a promoter with a TRE is injected into the oocyte nucleus, the DNA is replicated and concurrently assembled into chromatin in the presence of these receptors, thus allowing functional characterization of the receptors in a chromatin environment (23). Using such a system, we have found that both *Xenopus* TRαA and TRβA can activate efficiently a TRE-containing promoter as heterodimers with either *Xenopus* RXRa and RXRγ (22). We report here that to our surprise, *Xenopus* TRαB functions poorly as an activator. Furthermore,
the TRβ-BRXR heterodimer has a drastically reduced affinity for a TRE. We demonstrate that the defect in TRβ function is the result of mutations in the D domain located between the DNA and hormone binding domains in the previously reported TRα sequence, which was apparently derived from a mutant TRβ gene. Our experiments discovered that two amino acid residues in the D domain are critical for DNA binding, but not for heterodimerization or transcriptional activation.

MATERIALS AND METHODS

Construction of the Mutants—The coding regions of Xenopus TRα and TRβ (15) were cloned into the pSP64 vector (Promega) through a series of restriction and ligation. Mutants I–IV were made by switching domains between TRα and TRβ cloned into the pSP64 vector. To construct mutant I, a DNA fragment was restricted out of TRα with EcoRI (site present in the vector) and NsiI (in the insert) and cloned into similarly restricted TRβ vector. Mutant II was constructed by inserting the NcoI (in the vector/βIII) in (the insert) fragment of TRβ plasmid into TRβ plasmid. Mutant III contained the Bcl/I/III fragment of TRβ cloned into the TRα plasmid, and mutant IV contained the NsiI/II fragment of TRβ cloned into the TRα plasmid. Other mutants (V–XI) were constructed through PCR mutagenesis. First round of PCR was performed with two sets of primers: one (forward or reverse) containing the mutations and the other located at the very 3’ (reverse primer C: 5’-GGAGGCTTCTCCGTTGTGGCAGAA-3’) or 5’ (forward primer X: 5’-AGAAGCTTCTAGGATCATGAGAAGA-3’) end of the coding region of TRα gene. Primers containing mutations were as follows: for mutant V, forward primer E: 5’-GGAGGCTTCTAGGATCATGAGAAGA-3’ and reverse primer F: 5’-CTCAGGTCAAGTTAGCTCTGGA-3’; for mutant VI, forward primer G: 5’-GGAATTCGCCGCGGATCCGGTACG’ and reverse primer H: 5’-GCGTACCCGGCTCTGAGTGGC-3’; for mutant VII, forward primer K: 5’-GCAATCGCGGCGGAGGAAAAGA-3’ and reverse primer L: 5’-TCTCT-TCCGGCCGGGCGTACG’-3’. In all above PCRs, TRα was used as a template. For mutant IX, mutant III was used as a template and PCR was performed with E and F primers. Aliquots of above PCRs were mixed together and used as a template in the second round of PCR, performed with primers X and Y. DNA fragments containing mutations were restricted with NsiI/βIII (mutants V, VIII, X, and XI) or KpnI/βIII (mutants VI and IX) and cloned into TRα/pSP64 restricted with the same enzymes. Mutant VII was constructed by substituting mutant IX with RecBamHI enzymes, and DNA containing the mutations of interest was cloned into similarly cut pSP64-TRα vector. All mutants were verified by restriction analysis and sequencing.

Protein Overexpression in Oocytes—Stage 6 oocytes were injected with 200 ng/µl (5 ng/oocyte) mRNA encoding TRβ mutants and/or RXRs or RXRα as described (23). After overnight incubation at 18°C, protein extracts were prepared using 10 µl/oocyte of the protein isolation buffer (70 mM KCl, 20 mM HEPES, pH 7.5, 1 mM DTT, 5% glycerol, 1 mM EDTA, 0.1% Nonidet P-40, and a mixture of protease inhibitors, including phenylmethylsulfonyl fluoride, aprotinin, pepstatin, and leupeptin). Protein extracts equivalent to 2 oocytes were electrophoresed on a 10% SDS-PAGE gel and analyzed by Western blot with anti-TR and RXR antibodies (KPL, Kirklin, KY).

Gel Mobility Shift Assay—Identical amounts of each TRα mutant (usually 1 µl of the oocyte protein extract was diluted 20 times with the protein isolation buffer, and 2 µl of this diluted extract was used/sample) were mixed with 500 ng of dl-dc and 15 µl of the binding buffer A containing 20 µM Tris, pH 7.5, 5 mM MgCl₂, 100 mM KCl, 5 mM DTT, 0.1% Triton X-100, 10% glycerol, and a mixture of protease inhibitors (for DNA binding by homodimers) or binding buffer B containing 20 µM Tris, pH 7.5, 50 mM KCl, 2 mM DTT, 0.1% Nonidet P-40, 6% glycerol, and a mixture of protease inhibitors (for DNA binding by homo- and heterodimers). After 15–20 min of incubation on ice, 1 ng of synthetic, end-labeled double-stranded oligonucleotide bearing the TRE from Xenopus TRβ gene (xTRE) (24) and 20 × non-specific (mutated TRE or mTRE) xTRE (20 × 20 bp of TRα) specific competitor (xTRE) were added, where indicated. Samples were incubated at room temperature for an additional 20 min and analyzed on 5% native gel (at 150 V, for 140 min; Refs. 21 and 24).

Co-immunoprecipitation—This was done similarly as described (24). Oocytes were injected with mRNAs as described before, and incubated overnight in the presence of [35S]methionine. Protein extracts were made as before, except that the isolation buffer was supplemented with 0.5 µg/ml puromycin and X-100. Extracts were divided into two portions. One was used for Western blot analysis with TR and RXR antibodies to determine the levels of overexpressed proteins. The second aliquot (usually equivalent of 5 oocytes) was adjusted to 200 µl with the protein isolation buffer, and 5 µl of preimmune serum or serum containing the anti-TR antibody were added. Samples were incubated for 1 h at 4°C with gentle shaking, then 20 µl of protein A-Sepharose was added and the incubation was continued for another hour. Supernates were washed three times (10 min each wash) with the protein isolation buffer and three times (10 min each) in the same buffer in which the 70 mM KCl was replaced by 0.5 M NaCl. After removal of the last wash, 50 µl of 1 × sample buffer (37.5 mM Tris, pH 8.0, 2.5% β-mercaptoethanol, 1% SDS, 7.5% glycerol, 0.005% bromophenol blue) was added to the beads and the mixture was boiled for 5 min. The mixture was spun down, and 10–20 µl of the supernatant was analyzed on a 10% SDS-PAGE gel. The gel was dried and autoradiographed to detect the [35S]-labeled proteins.

Transcription Activation—This was done similarly as described by Wong et al. (22). Mutant TRβs and/or RXRs mRNAs were co-injected into oocyte cytoplasm (0.125 ng of each mRNA/oocyte). After 2–3 h of incubation at room temperature, single-stranded plasmid DNA containing a thyroid hormone (700 µl/oocyte) was added to the mixed sample and incubation was continued for another 8 h. After the incubation, the oocytes were homogenized in 0.25 M Tris, pH 7.5 (10 µl/oocyte), and divided into two aliquots. One aliquot (10–12 oocytes equivalent) was mixed with 500 µl of RNase-free H₂₀ (0.3 μg/oocyte). To 7 µl of the above RNA, 1 µl of 200000 cpm [32P]-labeled antisense CAT primer (5’-GGTGGTATACCGGATTTTCTCCTCAT-3’; 1 ng/µl) was added together with the annealing buffer (final concentrations were 0.4 mM CaCl₂, 20 mM Tris-HCl, pH 8.0). The mixture was denatured at 65 °C and then gradually cooled down to room temperature (1 h). To each sample, 1 µl of RNasin (40 units/µl, Promega), 1 µl of 25 mM dNTPs, 1 µl of 100 mM DTT, 2 µl of 2 mg/ml actinomycin D, and 100 units of Superscript II reverse transcriptase (Life Technologies, Inc.) were added together with 8 µl of 5 × reverse transcription buffer containing 250 mM Tris-HCl, pH 8.0, 30 mM MgCl₂. The reaction was brought up to 50 µl with H₂₀. After a 1.5-h incubation at 42 °C, the resulting DNA was ethanol-precipitated and analyzed on a 5% sequencing gel (National Diagnostic).

To determine the amount of promoter DNA injected into the oocytes, the remaining half of the oocyte homogenate was mixed with equal volume of 30 mM EDTA, 20 mM Tris-HCl, pH 7.5, 1% SDS. Samples were treated with proteinase K (800 µg/ml) for 1 h at 37°C, phenol/chloroform-extracted twice, ethanol precipitated with 0.5 M NaCl, and the DNA was resuspended in 100 µl of TE (10 mM Tris, pH 7.6, 1 mM EDTA) and treated with RNase A for 1 h at 37°C. The DNA was re-precipitated with ethanol and suspended in H₂₀ (4 µl/oocyte). NaOH was added to 0.1 M to denature the DNA. The DNA was slot-blotted onto a nitrocellulose membrane and fixed by UV irradiation (1200 mJ). Hybridization was done overnight at 42°C with a probe made of the CAT promoter vector as described (24).

Cloning and Sequence Analysis of Wild Type TRαβ DNA in X. laevis—Total RNA isolated from stage 63 (39) tadpoles treated for 24 h with thyroid hormone (40) was reverse-transcribed using a primer that is common to both TRα and TRβ gene and located near the carboxyl end of the coding region. A 5'-region encoding amino acids 62–186 and 3'-region encoding amino acids 335–414 (15) were PCR-amplified using primers common to both TRα and TRβ. The 5'-region (amino acids 70–186) and 3'-region (amino acids 353–420) were then cloned into pBluescript KS(--) (Stratagene). Five clones of the 3'-region were randomly isolated and sequenced, two of which were derived from TRα and one of which were from TRβ gene, based on DNA sequence comparison with the published sequences. For the 5'-region, the clones were hybridized with primers specific to either TRα or TRβ (located at amino acids 95–100 as described previously; Ref. 41). Four TRβ and three TRα clones were thus identified and sequenced.

Cloning and Sequencing of Genomic TRα Clones—A λ genomic library of homologous X. laevis DNA (41) was screened with a 370-base pair (b.p.) DNA probe of TRα B (amino acids 62–186), a region that is highly conserved between TRα and TRβ (15). The hybridization was performed overnight at 42°C in the buffer containing 50% formamide, 5 × Denhardt’s solution, 5 × SSPE, 0.2% SDS, and 100 µg/ml denatured salmon sperm DNA. Filters were washed three times (5 min each wash) at room temperature, in the buffer containing 2 × SSC and 0.2% SDS and subsequently 2 × 20 min at 65°C, in the buffer containing 0.25 ×
RESULTS

To study the transcriptional properties of Xenopus TRs, especially as heterodimers with RXRs, we have recently established a thyroid hormone (T3)-responsive in vivo transcriptional system by introducing exogenous TRs and RXRs into Xenopus oocytes through microinjection of their mRNAs into the cytoplasm (21, 22). As a reporter, we used the T3-responsive promoter of Xenopus TRβA gene fused to a CAT gene fragment (22, 24). Using such a system, we found that TRβB is a much weaker transcription activator than TRαA (see below). The experiments below were directed at determining the cause underlying the defects.

Two Residues in the D Domain of Xenopus TRαB Cause Severe Reduction in TRE Binding Affinity—The Xenopus TRαA and TRαB are highly homologous to each other (15). The full-length proteins differ only at seven amino acid positions (Fig. 1A). Five of the amino acid changes, three of which alter the charge of the protein, are located in the D domain between the DNA and hormone binding domains. Another is found in the NH2-terminal part of the protein, and the seventh one is localized to the hormone binding domain of the receptor. This last amino acid difference, an arginine in TRαA versus a cysteine in TRαB, is likely to be responsible for the 3-fold reduction in T3-binding affinity of TRαB (about 0.084 nM, Kd) compared with TRαA, TRβA, or TRβB (about 0.025 nM, Kd). To investigate which amino acid substitution is responsible for the defect in transcriptional activation by TRαB, the corresponding seven residues in TRαA were replaced either individually or in different combinations with those from TRαB (Fig. 1B). Particular focus was on the D domain, where the changes in charged residues are likely to affect DNA binding due to their proximity to the DNA binding domain. Thus, all combinational mutants of the double substitutions involving the charged residues were constructed for DNA binding and transcriptional activation studies.

To study the DNA binding properties of mutant TRs, we overproduced functional TRs and RXRs by microinjecting their mRNAs into the cytoplasm of the Xenopus oocytes. This method was chosen over the protein expression in Escherichia coli because TRs produced in the latter case were largely insoluble (24) and lacked potential post-translational modifications present in eucaryotic cells.

When equal amounts of the mRNAs for TRαA, TRαB, and the TR mutants were injected into Xenopus oocytes, essentially identical amounts of the different receptors were synthesized after the overnight incubation (Fig. 2A). Whole oocyte protein extracts containing these TRs were then mixed with similarly prepared extracts containing Xenopus RXRs (21) and used to study their DNA binding properties. A double-stranded TRE-containing oligonucleotide derived from the Xenopus TRβA promoter (xTRE; Ref. 24) was end-labeled and used with the oocyte extracts in the gel mobility shift assay. As expected, the TRαA-RXR heterodimer formed a strong complex with xTRE (Fig. 2B). In contrast, TRαB extract showed only a very weak binding. Among the TR mutants, all of those involving substitutions other than V133D and/or N149D (e.g. mutants I, II, III, VI, and VIII) showed wild type DNA binding affinity (Fig. 2B). In stark contrast, all mutants containing either the V133D or/and N149D changes produced little TR-RXR-TRE complex, indicating that the introductions of a negative charge at either one of these two positions interferes with TRE binding by TR-RXR heterodimers. Interestingly, the Arg → Cys substitution had no effect on DNA binding, even though it resulted in a loss of a positive residue that is present in other TRs (15). In addition, the mutant IV exhibited slightly but reproducibly stronger binding to the TRE than TRαB. This mutant differs from TRαB only at positions 47 and 392, both of which do not affect DNA binding when mutated individually (mutants I and II). Thus, in addition to charges, other factors can also influence DNA binding by TRs.

Three types of RXRs exist in Xenopus as in other species (25, 26). To test whether any mutations above affect RXR subtype-specific interaction with TRs, we compared TRE binding as heterodimers with Xenopus RXRα and RXRγ. Identical results were observed when either RXRα or RXRγ was used in the gel mobility shift assay with the TRs (Fig. 2C and data not shown), indicating that these mutations do not affect any RXR subtype.
specific contacts with TRs, if they exist.

To test the DNA binding specificity of the receptors, competition was performed with the wild type TRE (xTRE) itself or with the mutated TRE (mTRE), the mutations in which abolished both the response of the promoter to T3 and binding to TRzRXR heterodimers (22, 24). The binding by all TRs, including those that bind only very weakly, was found to be specific. Thus, the binding was efficiently competed out by xTRE itself but not by mTRE (Fig. 3A). In addition, competition was also done with two other known TREs: the TREp, a palindromic TRE consisting of two inverted repeats of the sequence AGGTCA, and TREgh, the TRE found in the human growth hormone gene, which is neither a palindromic TRE nor a TRE of two direct repeats of AGGTCA as in xTRE (27). Again, the complexes formed by xTRE and heterodimers of IXRz and all TR mutants showed similar competition profiles (Fig. 3B). The xTRE was found to be the strongest competitor and TREgh the weakest, similar to those observed for mammalian TRs and
the RXRα that the failure of TRβ, mRNAs for TRs and RXRα with RXRs. DNA, but not the capacity to form heterodimeric complexes efficiently is due to a reduction in their intrinsic ability to bind RXRs. The binding assay was done as in Fig. 2A for heterodimers except that the RXRα extract was omitted. Note that the pattern of complex formation by different receptors was the same as in Fig. 2A except the intensities of the complexes were much weaker (compare the signal of the specific complex relative to the nonspecific complex labeled by an asterisk and that of the free DNA). The mobilities of the complexes formed by TRs alone were comparable with those when RXRα was also present, suggesting that they were formed by TR homodimers.

**Xenopus TRβ** (24, 27, 28).

**Heterodimer Formation with RXRα Is Unaffected in TRαB and TRα Mutants**—The defect in DNA binding observed above for some TR mutants could be due to reduced ability to form heterodimers with RXRs or a decrease in TRs’ intrinsic affinity for the TRE or both. To test these possibilities, gel mobility shift assay was performed under reduced stringency conditions using only extracts containing TRs. The result demonstrated that TRαA could bind to xTRE in the absence of any RXR (Fig. 4). However, as reported for mammalian TRs (6–8), the binding by TRαA was much weaker than the TRαA-RXRα heterodimer (compare the signal ratio of the complex to the free TRE in Fig. 4 with that in Fig. 3B). The mobility of the complex suggest that it was formed by a TRαA homodimer. Like the corresponding heterodimer, the TRαB formed a homodimeric complex with the xTRE with the drastically reduced affinity. Similarly, all of the mutant receptors that were defective in binding as heterodimers with RXR were weak in binding as homodimers (TRαIV, V, VII, IX, X, and XI). On the other hand, all the mutants that could bind xTRE strongly as heterodimers formed the homodimeric complex with xTRE as efficiently as TRαA (TRαI, II, III, VI, and VIII) (Fig. 4). These results suggest that the failure of TRαB and some of the mutants to bind xTRE efficiently is due to a reduction in their intrinsic ability to bind DNA, but not the capacity to form heterodimeric complexes with RXRs.

To directly investigate the TR-RXR heterodimer formation by the TRs, mRNAs for TRs and RXRα were coinjected together with [35S]methionine into oocytes. After overnight incubation, protein extracts were prepared and immunoprecipitated with either anti-TR or anti-RXR antibody. SDS-PAGE gel analysis of the immunoprecipitates showed that anti-TR antibody precipitated not only TRs, but also RXRα and TRαA, or TRαB mRNA, and incubated in the presence of [35S]methionine. Oocyte extracts were precipitated with the TR antibody and analyzed by gel electrophoresis, showing that both TRαA and TRαB but not RXRα (upper panel) were precipitated by the antibody. However, Western blot clearly showed that RXRα was efficiently translated in the oocytes (lower panel).

**FIG. 4.** The relative binding affinities for xTRE by TRαA, TRαB, and the mutants alone are the same as their heterodimers with RXRα, except that the absolute affinities are much weaker than those by the corresponding heterodimers. Note also that the 35S label in the receptors was too light to produce a detectable signal during the short exposure time (5–10 s) used for the Western blot analysis. A, RXRα alone cannot be immunoprecipitated with the TR antibody. Oocytes were injected with no mRNA (CONTROL), RXRα, TRαA, or TRαB mRNA, and incubated in the presence of [35S]methionine. Oocyte extracts were precipitated with the TR antibody and analyzed by gel electrophoresis, showing that both TRαA and TRαB but not RXRα (upper panel) were precipitated by the antibody. However, Western blot clearly showed that RXRα was efficiently translated in the oocytes (lower panel).

**FIG. 5.** All TR mutants can form heterodimers with RXRα with equal efficiencies. A, the mRNAs for RXRα and various TRs were coinjected into the cytoplasm of oocytes and the oocytes were incubated in the presence of [35S]methionine. After overnight incubation, the protein extracts were prepared and either immunoprecipitated (IP) with an anti-TR antibody followed by analysis on a SDS-protein gel (upper panel) or analyzed by Western blots with the RXRα or TR antibody (lower panel). Note that Western blots indicated that equal amounts of TR and RXR were present in all samples except for the control oocyte without mRNA injection. While for an unknown reason, different TRs were not immunoprecipitated with equal efficiency, the relative ratios of TR to RXRα in different samples were about the same, indicating equal formation of TR-RXR heterodimers by different TRs. Note also that the 35S label in the receptors was too light to produce a detectable signal during the short exposure time (5–10 s) used for the Western blot analysis. B, RXRα alone cannot be immunoprecipitated with the TR antibody. Oocytes were injected with no mRNA (CONTROL), RXRα, TRαA, or TRαB mRNA, and incubated in the presence of [35S]methionine. Oocyte extracts were precipitated with the TR antibody and analyzed by gel electrophoresis, showing that both TRαA and TRαB but not RXRα (upper panel) were precipitated by the antibody. However, Western blot clearly showed that RXRα was efficiently translated in the oocytes (lower panel).
oocytes injected with no mRNA or only RXRα mRNA (Fig. 5B, lanes 1 and 2), demonstrating the specificity of the antibody. These results thus clearly indicate that TRαA, TRαB, and all TR mutants formed heterodimers with RXRα with equal efficiency. This conclusion was also supported by co-immunoprecipitation with an anti-RXRα antibody (data not shown).

**TRs That Are Defective in DNA Binding Are Also Weak Transcription Activators.** To compare the transcriptional activity of TRαA, TRαB, and the TR mutants, they were introduced into oocytes together with RXRα by injecting the corresponding mRNAs into the cytoplasm. After 2–3 h of incubation to allow the synthesis of the receptors, a single-stranded plasmid DNA containing the T3-responsive promoter of TRβA gene (24) was microinjected into the nucleus and the oocyte was incubated overnight to allow the conversion of the single-stranded DNA into the double-stranded and chromatized form, and the subsequent transcription from the resulting template in the presence or absence of T3. The transcription from the TRβA promoter was detected in oocytes both in the absence and in the presence of T3 in control oocytes without injected TR and RXR mRNA (Fig. 6A, lanes 1 and 2; Ref. 21). TRαA alone slightly activated the promoter in the presence of T3 (lanes 3 and 4) and this activation was drastically enhanced when RXRα was present (compare lanes 10 and 4). In contrast, no effect on the TRβA promoter was observed in the presence or absence of T3 when either TRαB (Fig. 6A, lanes 5 and 6) or RXRα (Fig. 6A, lanes 7 and 8) was individually present. While co-introduction of TRαB and RXRα was able to activate the promoter in the presence of T3, this activation was much weaker compared with that for TRαA and RXRα (Fig. 6A, compare lanes 12 and 10). Among the mutant receptors, those that could bind the xTRE as efficiently as TRαA (TRαI, II, III, VI, and VIII) activated the promoter to a similar extent as TRαA (Fig. 6A, lanes 25–34). All the other mutant TRs, which were defective in DNA binding, were found to be weak activators of the promoter (Fig. 6A, lanes 13–24).

The relative transcriptional activity of the TRs did not correlate perfectly with their binding affinity for xTRE in vitro. For example, the mutant TRαVII, involving a single amino acid substitution compared with TRαA (N149D), showed no detectable TRE-binding in the gel mobility shift assay. It, however, could activate the promoter to as much as half of the level.

**Fig. 6.** TRs that are defective in DNA binding are also weak transcription activators. A, oocytes were injected with TRs and/or RXRα mRNAs as indicated. Following 2–3 h of incubation, the single-stranded, TRE-containing promoter plasmid was injected into the nuclei of the oocytes. Half of the oocytes were incubated in the absence (−) and the other half in the presence (+) of T3. The transcription (TRβ RNA) from the plasmid was assayed using the primer extension method. An endogenous oocyte RNA (Internal control) was also detected, which served as an internal control for the primer extension. The plasmid DNA was also recovered from the same oocytes and found to be equal among different samples by slot-blot hybridization. The relative TRβ RNA levels were determined by normalizing the primer extension signals over the DNA recovery signals. Note that the defective DNA binders (TRαB, TRαIV, TRαV, TRαVII, TRαIX, TRαX, and TRαXI) were all weaker activators compared with the rest and that TRs alone were not as effective in transcription activation as when RXRα was also present. The transcription signals in absence of T3 were too weak to be visible in the plot of relative TRβ RNA levels. B, when the same TR mRNAs used in A were injected into oocytes and assayed for TR levels, equal amounts of various TRs were produced.
ever, due to the overall high level of sequence homology between
Xenopus TRα and TRβ, these earlier studies could not differentiate the two genes. To test whether both TRαA and TRαB are expressed during metamorphosis, the receptor cDNA was amplified from metamorphosing tadpoles by PCR using primers common to both TRαA and TRαB. The PCR products were analyzed by Southern blot hybridization using oligonucleotide probes specific to either TRαA or TRβ. The results showed the TRαA and TRβB were expressed at similar levels at the climax of metamorphosis (data not shown).

It is puzzling why Xenopus expresses both a functional and a dysfunctional TR at a time when TR is critical for development. This prompted us to ask whether the previously cloned TRβB was derived from the wild type gene or not. In addition, the possibility of a PCR artifact exists as the original clone was obtained by PCR (15). Therefore, total RNA from metamorphosing tadpoles was reverse-transcribed with a primer that is common to both TRαA and TRαB and located near the end of the coding region. Two regions of the cDNAs were PCR-amplified, again using primers conserved in the two genes, and cloned. The 5′ region encompassed amino acids 70–186, including the D domain between the DNA and hormone binding domains (15). The 3′ region covered amino acid 353–402, where an Arg → Cys substitution (amino acid 392, Fig. 1) in TRβ seemed to cause a 3-fold reduction in T3 affinity.5 Five clones of the 3′ region were randomly picked for sequence analysis. Three belonged to TRαB and two to TRαA based on sequence polymorphism other than at amino acid 392. All three TRαB clones had an Arg residue at amino acid 392 (data not shown), i.e. as in TRαA gene or a germline clones of TRβB gene (15). Thus, the wild type TRαB sequence is identical to TRαA in the hormone binding domain. The previously reported TRβC cDNA sequence (15) was either derived from a mutant TRαB gene or due to a PCR error at amino acid 392.

The clones of the 5′ region were identified by hybridization with primers located at amino acids 95–100 that were specific to TRαA or TRβB by making use of the polymorphic differences between the two genes. Four TRαB and three TRαA clones were isolated and sequenced. The sequences of new cDNA clones confirmed the polymorphic sequence differences at amino acids 95–100 between the two genes (data not shown). In the D domain, sequences of the new TRαB cDNAs had a Ser at amino acid 137 and Val at amino acid 151 (Fig. 8A), in agreement with the published TRβB cDNA sequence (15). The new TRαA clones had identical D domain sequences as previously reported except at amino acid 137, where an Ser was present in the new clones compared with a Gly in the original sequence. It is unclear whether this is due to polymorphism or a PCR artifact. However, as shown above, the TR with either an Ser or Gly at this position functioned identically.

Interestingly, at positions 133, 149, and 165 of the D domain, the amino acid residues in the new TRαB clones were found to be identical to those in TRαA. In the previously published Xenopus TRαB sequence, these positions differed in sequence from those in TRαA such that they resulted in a loss of a positive charge (position 165) and a gain of two negative charges (positions 133 and 149) compared with TRαA (15). While the loss of the positive charge had no effect on receptor function, the introduction of either negative residue or both drastically reduced the affinity for a T3RE and produced weak transcription activators as demonstrated above. Currently, it cannot be ruled out that PCR artifacts might be responsible for the previously published TRαB sequence. However, it is extremely unlikely to generate three mutations within a region of less than 100 base pairs, considering the high fidelity of the Taq polymerase used during the PCR cloning (-2 × 10⁻⁴ after a 10⁴-fold amplification; Ref. 29) and the fact that the nearby sequences in the DNA and hormone binding domains are the same in the previously published TRαA and TRαB sequences.
Thus, the results suggest that the previously published TRβ sequence was derived from a mutant TRβ gene in *X. laevis*. While several independent new TRαB cDNA clones confirmed the new TRαB sequence, these clones were obtained by PCR just like the previously published TRαA and TRαB. The numbers refer to the amino acid positions as previously reported (15). The asterisks and double asterisks in TRαB indicate the residues that were different between the previous reported TRαA and TRαB. The asterisks mark those residues which were found to be different in new cDNA clones compared with those of Yaoita et al. (15) and the double asterisks mark those residues in the new TRαB cDNA clone that are the same as those in Yaoita et al. (15), i.e., remain to be different from TRαA. Thus three amino acid residues were found to be mutated in the previously reported TRαB sequence in a 32-amino acid region. B, sequences of genomic TRαA and TRαB clones confirm the new TRα cDNA sequences. λ genomic clones were directly sequenced with an antisense primer. For a direct comparison with the cDNA sequences in A, the sequencing gel was presented upside down. Note that the genomic clones encode identical amino acids as the corresponding cDNA clones and that an intron/exon boundary is present at Leu^132.

**DISCUSSION**

We have provided evidence here that the previously cloned *Xenopus* TRαB gene is a mutant TR gene. Three residues in the D domain are altered in this mutant. Two of these mutations introduce negative charges into the receptor and are responsible for the defect in DNA binding. In addition, our combinatorial mutagenesis experiments suggest that in addition to primary sequence, secondary, and tertiary structures are important for both DNA binding and transcriptional activation.

**Structure-Function Correlations in DNA Binding**—Each of the three mutated residues in the mutant TRαB results in the loss of a positive charge (at position 165, Fig. 1) or gain of a negative charge in the D domain (at position 133 or 149). These mutations could be expected to affect charge-charge interaction between the receptor and DNA due to their proximity to the DNA binding domain. Interestingly, the Arg → Cys substitution at position 165, a position occupied by a positive amino acid in all other TRs, has no effect on DNA binding. The other two, both involving a neutral residue to aspartic acid substitution, cause severe reduction in DNA binding affinity.
The importance of D domain in DNA binding has also been demonstrated in two previous studies. In one case, Zechel et al. (30) reported that a deletion of a region of chicken TR whose encompassing the D domain strongly reduced its DNA binding ability. In the second case, Uppaluri and Towle (31) recently generated a library of mutant receptors for rat TRβ1 by PCR. Characterization of these mutants revealed a number of mutations in the D domain that affected DNA binding.

The structure of the D domain was recently reported in a crystal of a complex formed between a TR-RXR heterodimer and a TRE essentially identical to the x TRE used in our study. Numerous direct phosphate contacts formed mostly by the basic residues and water-mediated hydrogen bonds to the bases and phosphates in the D domain (positions 132–152) (32). In particular, the residues at positions equivalent to Val113 and Asn140 of Xenopus TRα, and/or their flanking residues make direct and/or water-mediated contacts with the negatively charged phosphate backbone. Assuming that TR structure is conserved, it would not be surprising that the introduction of a negative charge into either position or both inhibits DNA binding. On the other hand, the residue Arg145 is located within a disordered region in the crystal and this may not be important for DNA binding as we have observed here.

However, these simple amino acid-DNA interactions cannot explain the DNA binding defects for all mutants. For example, a single amino acid substitution at position 149 (Asn → Asp, mutant TRαVII) or at position 133 (Val → Asp, mutant TRαV) causes severe reduction in DNA binding ability (mutant TRαVII is the weakest DNA binder among all mutants). Double mutation of these two residues produces receptors with higher DNA binding affinities than those with only a single substitution. Similarly, the mutant TRαIV, which has all the substitutions in the D domain, but lacks two other substitutions (one in amino terminus and the other in the carboxyl terminus) present in the original TRαB mutant (Fig. 1A), binds DNA with a higher affinity than the original TRαB mutant, even though the substitution of the residue at the amino or carboxyl terminus alone does not influence DNA binding. These results suggest that the overall structure of the TR is an important aspect in DNA binding. These other amino acid residues, nearby or further away, are likely to play critical roles in secondary and/or tertiary structure formation, thus influencing receptor-DNA interaction.

**Effect of D Domain on Transcriptional Activation—** Our in vivo transcription assay clearly indicates that all mutant receptors can activate a TRE-containing promoter to varying degrees. This seems to contrast with the fact that some receptors have no detectable binding to a TRE in vitro. It is possible that these mutant receptors do have weak binding affinities for the TRE, but the complexes formed is too unstable to be detected by the gel mobility shift assay. Such weak, transient interaction with a TRE may be sufficient to activate the promoter in vivo. In this regard, it is interesting to note that essentially all D domain mutants generated by Uppaluri and Towle (31) that failed to bind DNA in vitro could nonetheless activate to varying degrees a TRE-containing promoter in cultured mammalian cells. On the other hand, all mutants that are defective in DNA binding are weak activators in oocytes, supporting a general correlation between DNA binding in vitro and transcriptional activity in vivo.

In addition to activating transcription in the presence of T3, TR-RXR heterodimers can also repress basal transcription in the absence of the ligand in oocytes (22). Unfortunately, under our in vivo transcription conditions, the basal transcription in the absence of the receptor is too low to be useful to study the transcriptional repressor activity of the unliganded receptors.

It is expected that the DNA binding defective mutants are weak repressors, as the repression activity is also mediated through a TRE (22, 24, 33–37).

In addition to influencing DNA binding, the D domain also plays a role in transcription activation by TRs. For example, while TRαVII, which contains a single substitution (V149D), is the weakest DNA binder, it is the strongest transcription activator among the mutants defective in DNA binding. The presence of additional mutations in the D domain in addition to this V149D substitution, e.g. in TRαX and TRαX, further inhibits the transcription activation by the TR, even though these additional mutations actually enhance DNA binding. Similarly, several other mutants (TRαV, TRαX, and the original TRαB mutant) can bind DNA better than TRαVII but are weaker activators in vivo. Thus, these other mutations in the D domain apparently affect transcription activation in a manner independent of their effects on DNA binding. Such an interpretation is also in agreement with two previous observations. Uppaluri and Towle (31) reported several mutants of the D domain generated by random PCR mutagenesis that had reduced transcription activation activities, but could bind TREs in vitro. Similarly, Lee and Mahdavi (38) identified by site-directed mutagenesis two basic regions (amino acids 142–144 and 196–198) that are important for transcription activation but not for DNA binding. The first region is flanked by the two amino acid residues that we have shown here to be important for DNA binding. Thus, the D domain of thyroid hormone receptors consists of intermixed subdomains critical for either DNA binding and/or transcription activation. The influence of the two functions of the receptor by the D domain is likely interrelated through structural regulation of the authentic DNA binding and activation domains.

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