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Gene-specific Changes in Promoter Occupancy by Thyroid Hormone Receptor during Frog Metamorphosis

*IMPLICATIONS FOR DEVELOPMENTAL GENE REGULATION**

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In all vertebrates, thyroid hormones (TH) affect postembryonic development. The role of the TH receptor (TR) in mediating the TH signal is complex as evidenced by divergent phenotypes in mice lacking TH compared with TR knock-out mice. We have proposed a dual function model for TR during development based on studies of frog metamorphosis. Here we examined an important assumption of this dual function model by using the chromatin immunoprecipitation assay, namely constitutive TR binding to promoters *in vivo*. We examined two target genes with TH-response elements (TRE) in their promoters, TRβ itself and TH/bZIP (TH-responsive basic leucine zipper transcription factor). By using an antibody that recognizes both TRα and TRβ, we found that TR binding to the TRβ promoter is indeed constitutive. Most surprisingly, TR binding to the TH/bZIP promoter increases dramatically after TH treatment of premetamorphic tadpoles and during metamorphosis. By using an antibody specific to TRβ, TRβ binding increases at both promoters in response to TH. *In vitro* biochemical studies showed that TRs bind TH/bZIP TRE with 4-fold lower affinity than to TRβ TRE. Our data show that only high affinity TRβ TRE is occupied by limiting levels of TR during premetamorphosis and that lower affinity TH/bZIP TRE becomes occupied only when overall the TR expression is higher during metamorphosis. These data provide the first *in vivo* evidence to suggest that one mechanism for tissue- and gene-specific regulation of TR target gene expression is through tissue and developmental stage-dependent regulation of TR levels, likely a critical mechanism for coordinating development in different organs during postembryonic development.

All vertebrates have two types of thyroid hormone receptors (TRs),[2] TRα and TRβ, that regulate gene expression by binding thyroid hormone (TH)-response elements (TREs) of TH-inducible genes and recruiting cofactors (1). In the absence of TH, TRs recruit corepressors, including N-CoR, SMRT, TBL1/TBLR1, HDAC3, and GPS2 (2–7). Corepressor binding is associated with deacetylated histones in the TRE region and gene repression. In the presence of TH, coactivators, such as SRC, p300, TRAP, and Mediator complexes, replace corepressors (8–11). Coactivator binding promotes transcription by acetylating histones and interacting with the basal transcriptional machinery.

The above knowledge based on *in vitro* studies complements extensive studies on the developmental role of TRs in frogs and TR knock-out mice. TR is important for postembryonic development of many organs (12). The destruction of larval organs and the formation of adult organs during frog metamorphosis is totally dependent upon TH (13, 14). Transgenic overexpression of mutant TRs and cofactors showed gene activation by TR is necessary and sufficient to initiate TH-dependent developmental transitions in frogs (15, 16). In knock-out mice lacking TRs, developmental defects are evident in brain, heart, and intestine, among other organs (17). Similar observations on the importance of TR in development have been noted in all vertebrates studied, including humans (18), fish (19), and chickens (20). Most interestingly, the phenotype of TR knock-out mice is dramatically different from mice lacking TH (17). Therefore, the study of the molecular mechanisms of TR in gene regulation *in vivo* is important for understanding the developmental actions of TR.

To bridge the gap between our knowledge of the developmental roles *in vivo* and the molecular mechanisms of TR action *in vitro*, we developed the dual function model for the role of TR during development (21, 22). Frog metamorphosis is a valuable model for studying hormonal control of postembryonic development because premetamorphic tadpoles naturally lack TH, and the large size of tadpoles allows direct study of the molecular mechanisms of gene regulation during development *in vivo*. In frogs, TRα is expressed throughout larval development, well before TH is synthesized and secreted into the blood, and both TRα and TRβ are expressed during metamorphic climax, characterized by extensive organ remodeling and high levels of TH (14). In the dual function model, during premetamorphosis TRα binds corepressors to deacetylate histones and down-regulate TH-response genes, while during metamorphic climax, TRα and TRβ activate those same genes by acetylating histones via binding coactivators in the presence of TH. *In vivo* support for this model comes from chromatin immunoprecipitation studies on TH-responsive promoters. Corepressor binding has been detected at TREs in premetamorphic tadpoles, and this binding is associated with low levels of acetylated histones (23–25). Conversely, coactivator binding with high levels of acetylated histones has been found during metamorphic climax (4, 26, 27).

A critical parameter for the dual function model is TR binding to TH-inducible genes *in vivo*. Even though ChIP assays suggest that TRs bind during both premetamorphosis and climax when TH is absent and present, respectively, developmental changes in TR expression hinted that levels of TR binding to TH-response promoters might vary dramatically during development. Here we used quantitative PCR (qPCR) to quantify TR binding to two different promoters during development, and we identified a contributing factor to differences we observed for TR binding between the promoters. These results expand our under-

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[2] The abbreviations used are: TR, TH receptor; TH, thyroid hormone; TRE, TH response elements; ChIP, chromatin immunoprecipitation; T_3, triiodothyronine; qPCR, quantitative PCR; bZIP, basic leucine zipper transcription factor; RXR, retinoid X receptor.

standing of tissue- and gene-dependent roles of TR during development.

MATERIALS AND METHODS

Animals and Antibodies—*Xenopus laevis* tadpoles and adults were reared in the laboratory or purchased from Xenopus I, Inc. Tadpoles at the indicated developmental stages (28) or premetamorphic tadpoles (stages 52–54) treated with 10 nM triiodothyronine (T_3) for 1–3 days at 18 °C with daily water changes without feeding were used for chromatin immunoprecipitation.

We used the following two rabbit antisera against TR: 1) anti-TR(PB) made by injecting full-length TRα but recognizes both TRα and TRβ (29), and 2) anti-TRβ made by coinjecting two synthetic peptides, REKRRKDEIQKSLVQKPEPT (amino acids 104–123 of TRβ synthesized on 8-MAP) and DRPGLASVERIEK (amino acids 290–302 of TRβ conjugated to keyhole limpet hemocyanin). Anti-ID14 antiserum made in rabbits immunized against the peptide ETKCRCNMDGDVE conjugated to 8-MAP (Invitrogen) was used as a negative control. ID14 is a novel extracellular protein expressed by intestinal epithelial cells (30).

Chromatin Immunoprecipitation—Chromatin was isolated from tadpole tails or intestines flushed with 0.6× phosphate-buffered saline. Organs were placed in 1 ml of nuclei extraction buffer (0.5% Triton X-100, 10 mM Tris-HCl, pH 7.5, 3 mM $CaCl_2$, 0.25 M sucrose, with protease inhibitor tablet (Roche Applied Science, Complete, Mini, EDTA-free), 0.1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride) in Dounce homogenizers on ice and crushed with 10–15 strokes using pestle A (Kontes). The homogenate was fixed in 1% formaldehyde with rotation at room temperature for 20 min, and the fixation was stopped with 0.1 M Tris-HCl, pH 9.5. The homogenate was centrifuged at 2000 × *g* at 4 °C for 2 min, and the pellet was resuspended in 1 ml of nuclei extraction buffer and re-homogenized in Dounce with 5–10 strokes using pestle A for tails and pestle B for intestines. Then the homogenate was filtered through a Falcon 100-μm cell strainer and centrifuged at 2000 × *g* at 4 °C for 2 min. The pellet was resuspended in 200–300 μl of SDS lysis buffer (Upstate Cell Signaling Solutions) on ice, sonicated to an average length of 800 bp, and centrifuged at 16,000 × *g* for 10 min at 4 °C. The chromatin in the supernatant was quantitated and frozen in aliquots at −80 °C.

For immunoprecipitation as reported previously (25), the DNA concentration of the chromatin was adjusted to 100 ng/μl using the SDS lysis buffer, and then diluted to 10 ng/μl with ChIP dilution buffer (Upstate Cell Signaling Solutions). After preclearing with salmon sperm DNA/protein A-agarose (Upstate Cell Signaling Solutions), input samples were taken, and 500 μl of each chromatin sample was added to tubes with anti-TR(PB), anti-TRβ, or anti-ID14 antibodies and salmon sperm DNA/protein A-agarose beads and incubated with rotation from 4 h to overnight at 4 °C. After incubation, the beads were washed with 1 ml of ChIP Buffer I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 50 mM HEPES, pH 7.5, 150 mM NaCl), Buffer II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 50 mM HEPES, pH 7.5, 500 mM NaCl), Buffer III (0.25 M LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0), and TE (10 mM Tris-HCl, pH 8.1, 1 mM EDTA, pH 8.0) in succession. After the last wash, 100 μl of elution buffer (0.5% SDS, 0.1 M $NaHCO_3$ (Sigma), 25 μg/ml proteinase K (Roche Applied Science)) was added to the samples and input controls and rotated at 65 °C for 6 h to overnight. The ChIP DNA was purified using the QIAquick PCR purification kit (Qiagen) and eluted with 40 μl of EB buffer (Qiagen, 10 mM Tris-HCl, pH 8.5). Analysis of ChIP DNA was done as described previously for radioactive PCR followed by gel electrophoresis and autoradiography (25) and quantitative PCR (16). Analysis of variance and post hoc tests of quantitative PCR results were carried out using Statview statistical software (Abacus Concepts, Berkeley, CA).

Gel Mobility Shift Assay—Receptor mRNA was made for TRαA, TRβAII, or RXRα contained in pSP64(poly(A)) (29, 31) using mMessage mMachine (Ambion). Large batches of oocyte extracts for TRα, TRβ, and RXRα were prepared by injecting 30–50 oocytes with 23 nl of 330 ng/μl receptor mRNA. After overnight incubation, surviving oocytes were homogenized in 20 μl of binding buffer per oocyte (20 mM HEPES, pH 7.5, 60 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 10% glycerol, 0.1% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride). Homogenate was centrifuged in an Eppendorf microcentrifuge at top speed at 4 °C for 10 min. Supernatant was collected and recentrifuged to remove lipids and debris. Aliquots were frozen at −80 °C. To ensure similar amounts of TRα and TRβ in the extracts, a preliminary experiment was done by injecting TRα and TRβ mRNA and metabolically labeling the proteins with [^{35}S]methionine. Oocyte extracts were then run on a gel and exposed to film to determine the amount of mRNA needed to produce equal amounts of TRα and TRβ by taking into account the number of methionines in each receptor.

Probes for the gel mobility shift assay spanned the single TRE in TRβ (31) and the two TREs in TH/bZIP (32) and had 4-bp overhangs on both 5′ and 3′ ends. The sequences were 5′-CGTCCTCCCTAGGCAGGTCATTTCAGGACAGCCCAGCGCCC and 5′-ACCAGGGCGCTGGGCTGTCCTGAAATGACCTGCCTAGGGAG for TRβ and 5′-ACTAGGGTTAAGTAAGGTGAATGCTCAGCCTCATTTGAACT and 5′-ACAGAGTTCAAATGAGGCTGAGCATTCACCTTACTTAACCC for TH/bZIP. Probes were annealed by adding 5 μl of 5 pmol/μl of each strand to Buffer III (New England Biolabs) in a 1.7-ml Eppendorf tube, putting the tubes in a beaker of boiling water, and allowing them to cool to room temperature. After ethanol precipitation, the pellet was dissolved in 12 μl of TE buffer, and the probe was labeled with T4 polynucleotide kinase in the forward reaction buffer in a 25-μl reaction (Invitrogen). After stopping the reaction and increasing the volume to 50 μl, unincorporated nucleotides were removed using a G-50 column (Amersham Biosciences) so that the final probe concentration was 0.1 pmol/μl.

Gel mobility shift assays were done based on procedures described previously (29, 31). Oocyte extracts (1–2 μl) from oocytes injected with RXRα and oocytes injected with either TRα or TRβ were mixed and incubated on ice for 10 min. The binding buffer (enough for a final reaction volume of 15 μl) and 1 μl of 2 μg/μl of sonicated poly(dI-dC) (Amersham Biosciences) in binding buffer was then added and incubated for 20 min at room temperature. 1 μl of 0.1 pmol/μl labeled probe and, as indicated, 3–15× unlabeled probe was then added and incubated for another 20 min. Gel mobility shift samples were run on a 6% PAGE in 0.5× TBE buffer (Invitrogen) at 250 V for 15 min. Gels were dried and exposed to film 15 min to overnight. Scatchard plot analysis was done using the public domain NIH Image program (developed at National Institutes of Health and available on the internet at rsb.info.nih.gov/nih-image) to determine the total counts bound and total counts free and then graphing bound *versus* bound/free in Statview (Abacus Concepts) to calculate the slope.

In Vitro Transcription and Western Blotting—The pSP64(poly(A)) vector (Promega) containing TRαA or TRβAII (29) was linearized with EcoRI and *in vitro* transcribed and translated following the manufacturer's protocol (TnT Quick Coupled transcription/translation system, Promega). Standard protocols for SDS-PAGE and protein transfer were performed, followed by Western blotting using anti-TR(PB) and anti-TRβ (38) antibodies and ECL-plus chemiluminescent detection (Amersham Biosciences).

RESULTS

TR Binding to TREs in Vivo Is Gene-specific and TH-dependent—Because TR function is critical for metamorphosis and vertebrate development in general, molecular mechanisms for regulating specific TH-response genes *in vivo* are of considerable interest. Based on *in vitro* studies, TR binds TREs constitutively, *i.e.* TRs bind TREs in the absence and presence of ligand (1). Most interestingly, in our studies on TR binding to TREs *in vivo* during metamorphosis by ChIP, we often observed variable amounts of TR binding to TRE of the TH-inducible TH/bZIP promoter, depending on the presence of ligand (15, 25). To investigate the TH-dependent phenomenon, we treated premetamorphic tadpoles with or without 3,5,3′-triiodothyronine (T_3), a potent form of TH, and analyzed TR binding to two TH-inducible promoters, TRβ and TH/bZIP, in the intestine and tail with anti-TR(PB) antibody (Fig. 1). TR binding remained constant at the TRβ promoter in the presence or absence of T_3 in both the intestine and tail (Fig. 1, *top panels*), and this constitutive binding was found at the TH/bZIP promoter in the intestine as well (Fig. 1, *lower panels*). However, an increase in TR binding was detected in the presence of T_3 in the tail (Fig. 1, *lower panels*).

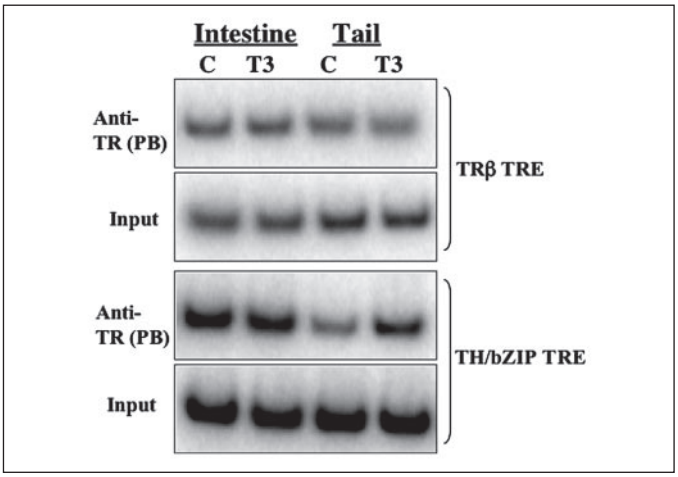

FIGURE 1. **ChIP assay with anti-TR(PB) antibody and conventional PCR and gel autoradiography.** ChIP assay using anti-TR(PB) antibody was performed using chromatin combined from three intestines or tails from premetamorphic control tadpoles (*C*) or tadpoles treated with 10 nM T_3 for 24 h (*T3*). TR binding to the TRβ and TH/bZIP promoters was detected by gel autoradiography following PCR amplification. For the TRβ promoter, it appears that there was little effect of T_3 treatment for both organs in TR binding, whereas there appears to be enhanced TR binding to the TH/bZIP promoter in the tail after T_3 treatment. *Input* shows approximately equal amounts of chromatin were used in the immunoprecipitation step.

To quantitate any potential differences in TR binding, we carried out qPCR. We increased the scope of the analysis by examining multiple time points of T_3-induced metamorphosis and at different stages during natural metamorphosis. For these qPCR experiments, we included a control antibody, anti-ID14, and a primer/probe set for a control DNA region, the TRβ exon 5. The control DNA region, exon 5 of the TRβ gene, does not contain a TRE and is at least 20 kb away from the promoter where the TRE is located (33). This controls for sufficient sonication of the chromatin in the assay. Thus, the very low amounts of precipitated exon 5 DNA, expressed as % input, for both antibodies, anti-TR(PB) and anti-ID14, indicate that the chromatin was sonicated sufficiently to avoid chromatin fragments containing both the TRE and exon 5. We suggest that this level of % input at exon 5 represents the background for the assay. We also found background levels of % input at the TH/bZIP and TRβ TREs with the anti-ID14 antibody, indicating the washing steps after immunoprecipitation effectively removed nonspecific interactions.

Consistent with the above analysis, TR binding to the TH/bZIP TRE was dramatically and statistically significantly increased in the presence T_3 in both the tail ($F_{3,10} = 7.9$, $p < 0.005$ (This *F*-statistic has 3 and 10 degrees of freedom reflecting treatment levels and sample sizes.)) and intestine ($F_{3,10} = 9.1$, $p < 0.003$), where the maximal levels of increase after T_3 treatment were about 10- and 7-fold, respectively (Fig. 2, *A* and *B*). Scheffe's post hoc tests revealed no differences in the immunoprecipitated TRE region as % input among T_3-treated samples. These same ChIP DNA samples were used to quantitate TRβ TRE immunoprecipitation, where less dramatic increases were observed, *i.e.* the immunoprecipitated TRE region as % input changed less than 2-fold in the tail and 2–4-fold in the intestine (Fig. 2, *A* and *B*) (TABLE ONE). This smaller change at the TRβ TRE was not detectable using conventional PCR analysis, and indeed, because of the differences in values when using chromatin isolated from multiple animals with different days of T_3 treatment, the changes caused by T_3 treatment in the tail were not

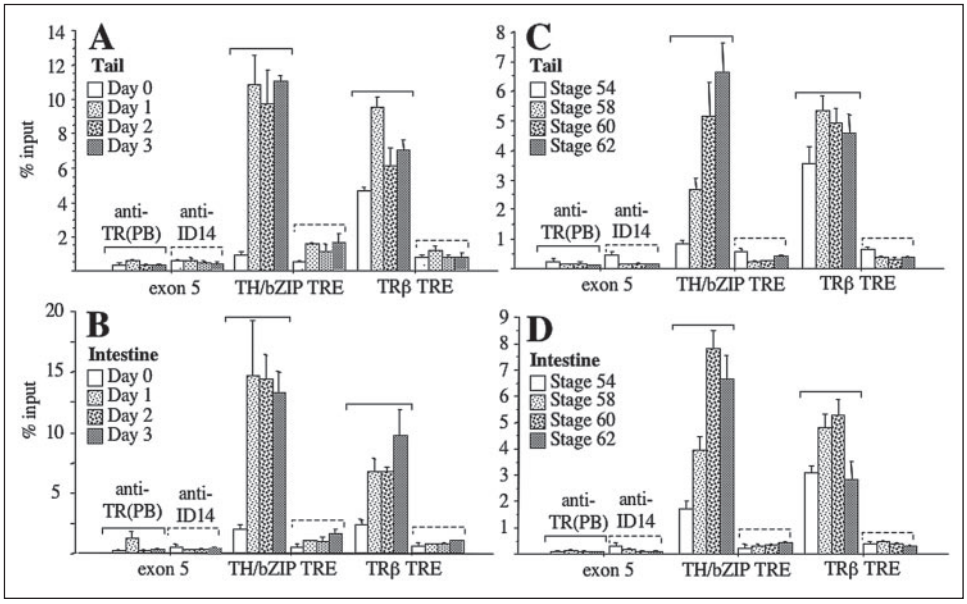

FIGURE 2. **Quantitative PCR analysis of TR binding to T_3-responsive promoters during T_3-induced and natural metamorphosis.** Two T_3-responsive promoters, TRβ and TH/bZIP, and a control DNA region, exon 5 of the TRβ gene, were analyzed for TR binding after immunoprecipitation with anti-TR(PB) antibody and a control antibody against an irrelevant protein (ID14) by using qPCR on the resulting ChIP DNA. *A* and *B*, chromatin was isolated from tails (*A*) and intestines (*B*) of premetamorphic tadpoles treated with 10 nM T_3 for 0 or 1–3 days and used in the ChIP assay. The *bars* represent mean ± S.E. from 2 to 6 replicate experiments performed on separate days, each using three tadpoles. Note the dramatic increase in TR binding in response to T_3 at the TH/bZIP promoter in the intestine and especially the tail. *C* and *D*, chromatin was isolated from tails and intestines of tadpoles at the indicated metamorphic stages and used in the ChIP assay. The *bars* represent mean ± S.E. from 3 to 9 replicate experiments, each replicate using three tadpoles, performed on separate days. Note the similar results as in *A* and *B*.

TABLE ONE

Increases in TR binding to TRβ and TH/bZIP TREs in the tail and intestine during T_3-induced and natural metamorphosis

Values represent the maximum fold increase in immunoprecipitated TRE as % input between tadpoles treated with or without T_3 and between premetamorphic (stage 54) and metamorphic tadpoles. Antibodies used were anti-TR(PB) (for both TRα and TRβ) and anti-TR (for TRβ). Calculations used the data for Fig. 2 and Fig. 7.

	T_3-induced metamorphosis				Natural metamorphosis			
	TRβ TRE		TH/bZIP TRE		TRβ TRE		TH/bZIP TRE	
	PB	β-specific	PB	β-specific	PB	β-specific	PB	β-specific
Tail	<2×	3×	10×	10×	<2×	5×	8×	20×
Intestine	3×	3×	7×	10×	<2×	2×	5×	10×

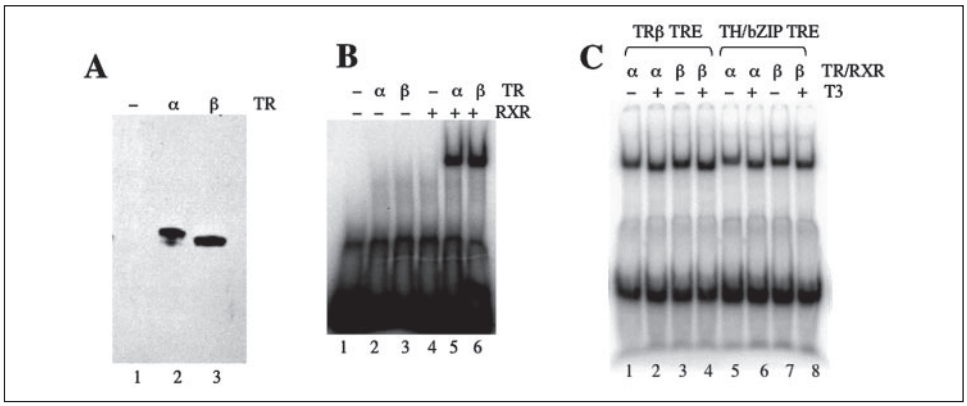

FIGURE 3. **Gel mobility shift assay showing requirement for the TR/RXR heterodimer and the lack of effect of T_3 in binding to TREs.** *A,* Western blot shows equal amounts of TRα and TRβ used in the gel mobility shift assays. Equal volumes of extract from oocytes injected with TRα or TRβ or no mRNA were loaded on an SDS-polyacrylamide gel, transferred to membrane, and immunoblotted with anti-TR(PB) antibody. *B,* both TR and RXR are required for efficient binding to the TH/bZIP TRE. When no receptor (*lane 1*) or TRα, TRβ, or RXR were added alone (*lanes 2–4*), no shifted band is detectable. However, when RXR and either TRα or TRβ were included in the reaction, a significant shift was observed (*lanes 5* and *6*). *C,* binding reactions with TRα/RXR or TRβ/RXR and labeled TRβ or TH/bZIP TREs were carried out with or without T_3. The presence of T_3 did not affect the amount of binding to TRβ or TH/bZIP TREs by either TRα/RXR or TRβ/RXR heterodimers, although mobility of the complex was increased slightly by T_3 (compare *odd lanes* to *even lanes*). This experiment was repeated with similar results.

statistically significant ($F_{3,10} = 3.3$, $p < 0.068$), although the increase in the intestine was significant ($F_{3,10} = 16.3$, $p < 0.0004$).

The results of TR binding during natural metamorphosis are by and large similar to TR binding results from T_3-induced metamorphosis (Fig. 2, *C* and *D*). We examined stages from beginning (stage 54) to climax of metamorphosis (stage 62), when maximal levels of T_3 are detectable (34) and two stages in-between (stage 58 and 60). Stage 58 is forelimb emergence and onset of intestinal remodeling; stage 60 is when the highest number of apoptotic epithelial cells is observed in the remodeling intestine (28, 35). At the TH/bZIP TRE, there was a gradual and significant increase in TR binding across development ($F_{3,22} = 10.3$, $p < 0.0002$ for tail and $F_{3,14} = 112.5$, $p < 0.003$ for intestine), up to 8-fold in the tail and 5-fold in the intestine (Fig. 2, *C* and *D*) (TABLE ONE). These increases became significant by stage 60 in both organs based on Scheffe's post hoc tests. At the TRβ TRE, no significant differences in TR binding across development were observed in the tail ($F_{3,22} = 1.5$, $p < 0.23$) or the intestine (post hoc tests showed no pairwise significant difference, although TR binding differences were significant overall, $F_{3,14} = 4.1$, $p < 0.028$).

TRα and TRβ Have a Higher Affinity for TRβ TRE Compared with the TH/bZIP TRE—A generality based on the above data is that the level of TR binding to the TH/bZIP TRE in tails and intestines of untreated, premetamorphic tadpoles was at or marginally above background levels, based on signal from exon 5 and control antibody. In addition, the TR binding at the TRβ TRE in premetamorphosis was much higher above background compared with the TH/bZIP TRE, especially in the tail. In the presence of T_3, an increase in TR binding was greater at the TH/bZIP TRE than at the TRβ TRE. These differences between TRβ and TH/bZIP TREs motivated experiments to determine the underlying basis for the differences between promoters. By using gel mobility shift assays, we tested several possibilities that may explain the differences as follows. 1) The T_3-induced conformational change in TR *per se* may increase its affinity for the TH/bZIP promoter. 2) TRα, which is the predominant TR before T_3 treatment, may have a lower affinity than TRβ for the TH/bZIP TRE. 3) TRs may have different affinities for TRβ *versus* TH/bZIP TREs.

For the gel mobility shift assays, we isolated frog oocyte cytoplasm containing TRα, TRβ, or RXR after injection of corresponding mRNAs. We adjusted mRNA concentration for production of the oocyte cytoplasm based on [^{35}S]methionine incorporation into the TRs after a pilot mRNA injection so that the isolated cytoplasms had equal amounts of the two TRs (data not shown), and we confirmed this result by Western blot using the same antibody as in the above ChIP experiments, anti-TR(PB), that recognizes both TRα and TRβ (Fig. 3*A*). Next, we showed this cytoplasm was competent in the gel mobility shift assays, where a shift was seen when TR, either α or β, and RXR were coincubated in the binding reaction and not when only one receptor or no receptors were included (Fig. 3*B*).

To address the simple possibility that there is some difference in the ability to bind the TH/bZIP TRE *versus* TRβ TRE in the presence or absence of T_3, we carried out a gel mobility shift assay that included T_3 (Fig. 3*C*). The results showed equal binding by both TRs to both TREs in the presence or absence of T_3. The slight difference in migration in the presence of T_3 is likely due to a conformational change in the TRs induced by T_3, causing different migration on the nondenaturing gels (36).

The other potential explanations for the low binding in TH/bZIP in the absence of T_3 may relate to binding affinity differences between the

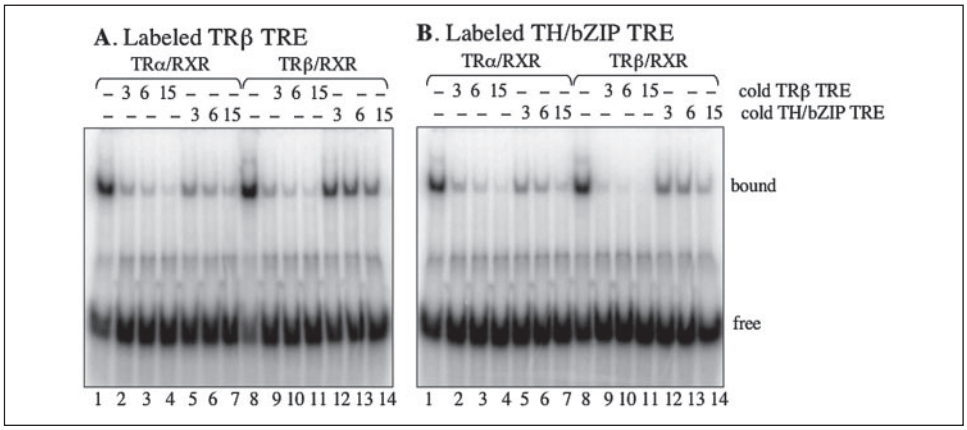

FIGURE 4. **Competition experiment between TRβ and TH/bZIP TREs for TR binding.** Labeled TRβ TRE (*A*) or labeled TH/bZIP (*B*) was used in these experiments. Competition of the labeled TREs for binding to TRα/RXR or TRβ/RXR was determined by including 3-, 6-, or 15-fold excess cold TRβ or TH/bZIP TRE. For both labeled TREs, cold TRβ TRE competed more effectively than cold TH/bZIP TRE for binding to TRα/RXR (compare *lanes 2–4* to *lanes 5–7*) or TRβ/RXR (compare *lanes 9–11* and *lanes 12–14*).

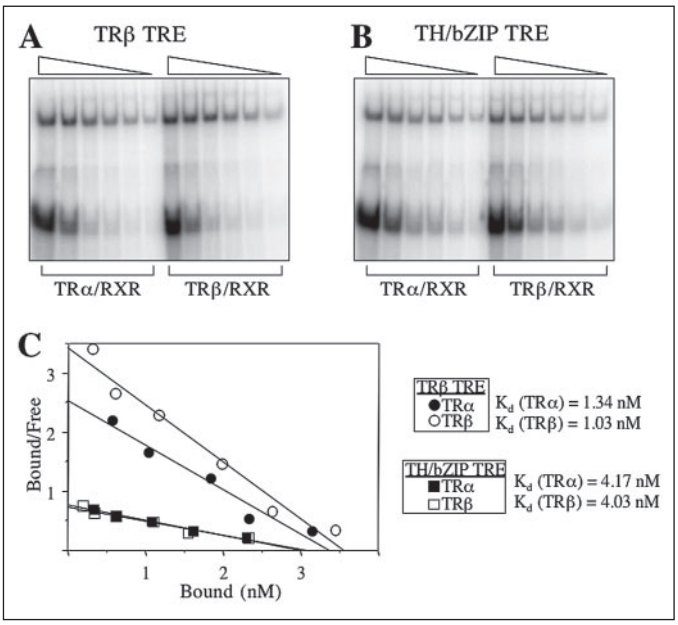

FIGURE 5. **Scatchard plot analysis of TR binding to TRβ and TH/bZIP TREs.** Labeled oligonucleotides for TRβ (*A*) or TH/bZIP (*B*) were added to the TRα/RXR or TRβ/RXR binding reactions in 2-fold serial dilutions starting from 0.2 pmol per reaction. Densitometry measurements of the bound and free probe in each lane were used in a Scatchard plot (*C*) to calculate the slopes of the line generated by bound/free *versus* bound plot to generate K_d values. Note that TRα/RXR binds each TRE with similar affinity as TRβ/RXR but that both TRs bind TRβ TRE with higher affinity than TH/bZIP TRE. Also, the similarity of the *x* intercepts, which indicates the total TR in the binding reaction, for the two TRs for either TRβ TRE or TH/bZIP TRE confirms similar amounts of TRα and TRβ in the oocyte extract used as the source of TR for the binding reactions.

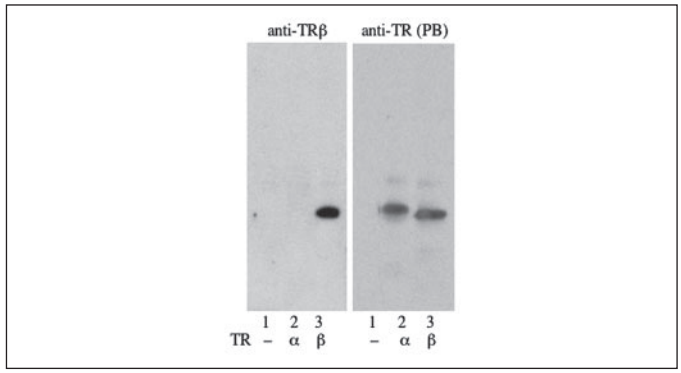

FIGURE 6. **Western blot showing specificity of anti-TR antibodies.** Anti-TR(PB) and anti-TRβ antibodies were used to probe membranes containing *in vitro* transcribed and translated TRα (*lanes 2*) and TRβ (*lanes 3*). *Lane 1* is unprogrammed reticulocyte lysate. Note that anti-TR(PB) antibody recognizes both TRα and TRβ, whereas anti-TRβ antibody recognizes only TRβ.

two TR isoforms or between TREs. First, we performed a TRE competition experiment where TR binding to radiolabeled TREs was competed with unlabeled TREs (Fig. 4). By using radiolabeled TRβ TRE, we incubated mixtures of TRα and RXR or TRβ and RXR with increasing amounts of cold TRβ TRE or TH/bZIP TRE (Fig. 4*A*). For both TRα and TRβ, cold TRβ TRE was a better competitor than cold TH/bZIP TRE for TR binding as shown by the greater reduction of bound radiolabeled TRβ TRE in the presence of cold TRβ TRE (Fig. 4*A*, compare *lanes 2–4* with *5–7* and *lanes 9–11* with *12–14*). Similar results were obtained with the reciprocal experiment using radiolabeled TH/bZIP TRE (Fig. 4*B*), showing that cold TRβ TRE again competed more efficiently than TH/bZIP TRE (compare *lanes 2–4* with *5–7* and *lanes 9–11* with *12–14*).

Even though both TRs bind the TRβ TRE more strongly than the TH/bZIP TRE, TRα may not bind the TREs as strongly compared with TRβ. In premetamorphic tadpoles, TRα is predominant, and TRβ expression is very low, whereas TRβ expression is up-regulated in the presence of T_3 (37). Thus, the low TR binding to TH/bZIP may be due to a potentially low TRα binding affinity for TH/bZIP TRE and low TRβ protein levels in premetamorphosis. To examine potential differences in binding to the two TREs between the TR isoforms, we performed a Scatchard plot analysis (Fig. 5). We used either radiolabeled TRβ or TH/bZIP TREs in decreasing amounts in the binding reactions with a constant amount of TRα or TRβ in a gel mobility shift assay (Fig. 5, *A* and *B*). These radiographs were used to determine the total amount of bound and free TRE from each binding reaction for the Scatchard plot analysis (Fig. 5*C*). The results corroborate data from the above competition experiments showing that for each TR the binding affinity is higher for TRβ TRE than TH/bZIP TRE with the K_d (dissociation constant) for TRβ TRE 4-fold lower than the K_d for the TH/bZIP TRE. In addition, both TRs had identical K_d values for either TRβ TRE or TH/bZIP TRE, indicating identical affinity for each TRE by TRα and TRβ.

Up-regulation of TRβ Expression by TH Leads to High Occupancy of TRβ at TREs—The increase in TR binding at TREs after T_3 treatment suggests that TR levels are not sufficient to saturate TREs, at least the weaker TH/bZIP TRE in premetamorphic tadpoles. As T_3 treatment preferentially induces TRβ expression (37), we would predict that TRβ binding at TREs would increase more dramatically than total TR binding to the TREs. To examine this hypothesis, we performed ChIP assay using a TRβ-specific antibody on tail and intestine during development. First, we generated a TRβ-specific antibody by immunizing a rabbit with two TRβ-specific peptides (see "Materials and Methods"). Western blot analysis of *in vitro* translated TRα and TRβ with this polyclonal

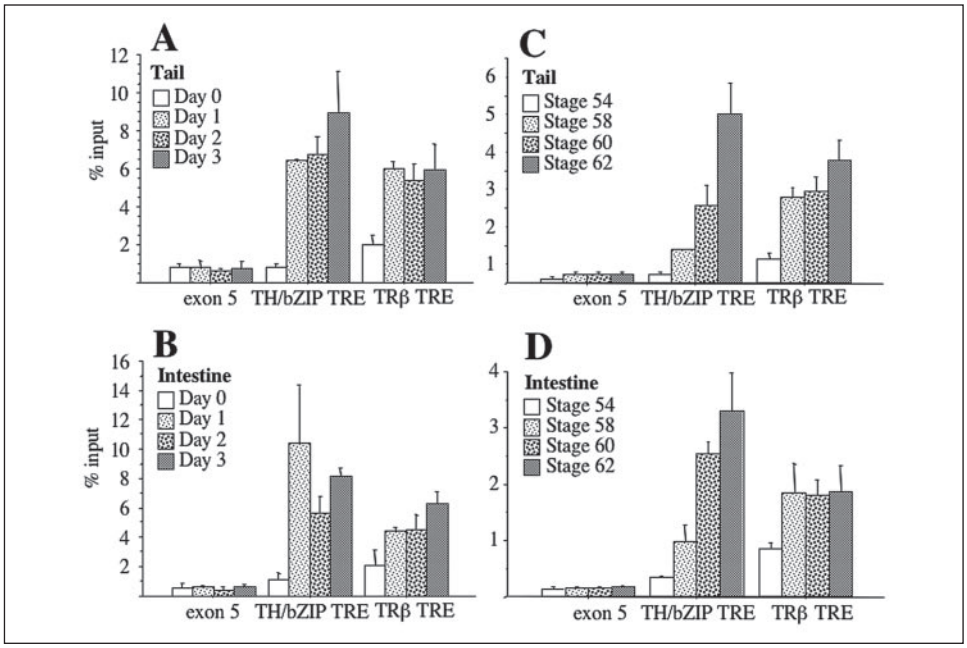

FIGURE 7. **Quantitative PCR analysis of TRβ binding to T_3-responsive promoters in T_3-induced and natural metamorphosis.** Two T_3-responsive promoters, TRβ and TH/bZIP, and a control DNA region, exon 5, were analyzed for TRβ binding after immunoprecipitation with anti-TRβ antibody by using quantitative PCR on the resulting ChIP DNA. *A* and *B,* chromatin was isolated from tails (*A*) and intestines (*B*) of premetamorphic tadpoles treated with 10 nM T_3 for 0 or 1–3 days and used in the ChIP assay. The *bars* represent mean ± S.E. from 2–5 replicate experiments performed on separate days, each using three tadpoles. Note the dramatic increase in TRβ binding in response to T_3 at both TREs in tail and intestine. *C* and *D,* chromatin was isolated from tails (*C*) and intestines (*D*) of tadpoles at the indicated metamorphic stages and used in the ChIP assay. The *bars* represent mean ± S.E. from 2 to 4 replicate experiments performed on separate days. Note the similar results as in *A* and *B*.

AGGTCA NNNN AGGTCA	consensus TRE
AGGTCA TTTC AGG**A**CA	TRβ TRE
GGGT**T**A ATGA AGGT**G**A	TH/bZIP TRE1
AG**T**TCA AATG AGG**CTG**	TH/bZIP TRE2

FIGURE 8. **TRE sequence comparison.** The *Xenopus* TRβ TRE has a single nucleotide difference from this consensus, whereas the TH/bZIP TREs have 3–4 differences (40). Differences are shown in *boldface*.

antibody, anti-TRβ, showed that it is specific for TRβ (Fig. 6*A*), even though similar amounts of TRα and TRβ were present as shown by the anti-TR(PB) antibody used above (Fig. 6*B*). When the ChIP assay was carried out with anti-TRβ antibody comparing control and T_3-treated tadpoles, TRβ binding increased by about 10-fold at the TH/bZIP TRE and 3-fold at the TRβ TRE in both tails ($F_{3,10} = 16.8$, $p < 0.0003$ for TH/bZIP and $F_{3,10} = 5.7$, $p < 0.015$ for TRβ) and intestines ($F_{3,8} = 7.8$, $p < 0.009$ for TH/bZIP and $F_{3,8} = 2.6$, $p < 0.12$ for TRβ) (Fig. 7, *A* and *B*). These increases were similar or slightly higher than those observed with the anti-TR(PB) antibody. During natural metamorphosis, TRβ binding to the TH/bZIP TRE gradually increased to 20- and 10-fold in the tail and intestines, respectively, from premetamorphosis to metamorphic climax (Fig. 7, *C* and *D*). At the TRβ TRE during natural metamorphosis, the increases were 5- and 2-fold in the tail and intestines, respectively. The level of TRβ binding was at background control antibody levels for the TH/bZIP TRE for both organs in premetamorphic tadpoles and was at or marginally above background levels for the TRβ TRE and increased much more during metamorphosis than that observed for anti-TR(PB) antibody (TABLE ONE). These results are consistent with the relative changes in TRα and TRβ expression during natural and T_3-induced metamorphosis (see "Discussion").

DISCUSSION

Because *in vitro* studies showed constitutive binding of TR to TREs independent of hormone (1), it was surprising to find increased recruitment of the TR to the TH/bZIP promoter after addition of exogenous TH *in vivo* given the significant levels of TRα expression in premetamorphic tadpoles (37, 38). At the same time, we found limited change in TR binding to the TRβ TRE in response to TH. The accuracy of these results was initially in doubt because it was based on conventional PCR and autoradiography, techniques of dubious quantitative value. Therefore, we carried out qPCR and confirmed promoter-specific, differential recruitment of TR to the two TREs. Next, we used gel mobility shift assays to investigate the underlying cause for the differences in TR binding between the two promoters *in vivo*. Our data showed that TRα and TRβ bind to the TREs with identical affinity and that the TH/bZIP TRE has a 4-fold weaker affinity than TRβ TRE for either receptor, a result consistent with a sequence comparison of the TRβ and TH/bZIP TREs with a consensus TRE (Fig. 8) (39). The TRβ TRE has a single nucleotide different from the consensus, whereas the two TH/bZIP TREs have 3 or 4 nucleotide differences each. Most interestingly, even though there are two TREs in the TH/bZIP promoter, only a single TR/RXR heterodimer is bound under gel mobility shift conditions (our data and see Ref. 40).

The combination of results from the ChIP and gel mobility shift assays suggests the following hypothesis explaining the differences in TR binding *in vivo* for the two promoters. TH/bZIP TRE occupancy by TRs is low in premetamorphosis because of limiting protein levels of TR, which allows the binding to the 4-fold higher affinity TRβ TRE-binding sites. Then, in T_3-induced or natural metamorphosis, even the lower affinity TH/bZIP TRE-binding sites would be occupied because of the T_3-induced expression of TRα and TRβ. Furthermore, the relatively little change in TR binding at the TRβ TRE implies that the TR expression levels in premetamorphosis are able to saturate these high affinity TRE-binding sites so that even during metamorphosis with higher levels of TR little binding occurs.

We examined a corollary of the above hypothesis that the TRβ isoform binding would be low at both promoters in premetamorphosis when TRβ expression was low and would increase at both promoters in the presence of T_3 after enough time had occurred for TRβ synthesis. We predicted this increase would be greater than the increase observed with the anti-TR(PB) antibody that recognizes both TR isoforms. Indeed, the increase for TRβ binding during natural metamorphosis was much greater than that seen for the other antibody at both promoters. Although the increase in TRβ binding compared with total TR binding was less dramatic during T_3 treatment, this result was consistent with the relative expression of TRα and TRβ during T_3 treatment. During natural metamorphosis, TRα mRNA expression increases only 2–3-fold in both the intestine and tail, whereas TRβ mRNA increases by over 10-fold (41–43). This leads to a much higher TRβ

to TRα ratio at climax than in premetamorphosis, thereby leading to a higher increase in TRβ binding to TREs than in total TR binding. In contrast, during T_3 treatment both TRα and TRβ increase dramatically at least in the tail (43); thus even though overall TR binding increased at both promoters, the relative proportions of TRβ might not increase significantly, as we observed.

The results of this study have strong implications for the role of TR in development. The dual function model for the role of TR states that TH-responsive promoters are repressed during premetamorphosis and activated during metamorphosis because of the critical role of ligand for TR function (21). No exceptions to this model have been identified so far regarding the up-regulation of direct response genes by T_3 during metamorphosis (22). However, our ChIP results show that not all T_3-response genes are bound by TR during premetamorphosis, indicating that TR does not regulate these genes before metamorphosis. Rather, the lack or low levels of expression of some TH-response genes before metamorphosis, such as TH/bZIP (41, 43), must be through TR-independent mechanisms via other transcription factors or formation of a repressive chromatin structure. Thus, our data indicate that the application of the dual function model during premetamorphosis cannot be universally applied to all T_3-response genes and needs to incorporate TRE affinity for TRs and changing levels of TR during development. During premetamorphosis, the dual function model applies only to those genes with high affinity TREs. As receptor levels increase continuously during development (37), more and more promoters containing weaker TREs come under positive regulatory control of TR and TH.

Another hypothesized mechanism for TR function in development is its autoinduction. Previous studies in frogs revealed that TH regulates its own receptors, and this autoregulation is thought to be important for the developmental role of TH because it is correlated with metamorphic progression. However, because of the lack of knock-out technology in frogs, this hypothesis has not been directly tested. Our results here and the kinetics of TRβ and TH/bZIP mRNA expression indirectly implicate a critical role of autoregulation. The kinetics of TRβ and TH/bZIP mRNA expression are different after T_3 treatment, where transcription of the TRβ gene is up-regulated to maximal levels well before that from the TH/bZIP gene, which exhibits a second wave of increase in mRNA levels after T_3 treatment (43). The initial rate of increase of TH/bZIP transcript may be limited by relatively low levels of TR binding at the promoter because of the lower affinity TRE, whereas after the start of T_3 treatment when sufficient TR synthesis has occurred, the TR binding to the TH/bZIP TRE may be increased enough to promote a boost in mRNA production. Lack of autoregulation would likely result in the inability of TR to induce genes important for metamorphosis, such as TH/bZIP, due to results from our ChIP showing low TR binding to TH/bZIP TRE before metamorphosis.

Amphibian metamorphosis involves complex coordination of different transformations at different developmental stages in various organs. Different TH-response genes may play roles at different stages of metamorphosis in different organs/tissues or even within a single tissue, and their expression levels would need to be controlled accordingly. For example, limb development occurs much earlier, at the onset of metamorphosis, than tail resorption, which is the last process to complete. One mechanism to control such developmental timing is to have tissue-specific TR expression levels for controlling tissue sensitivity, *i.e.* the ability to activate T_3-response genes (44). Specifically, the tissues first to transform during metamorphosis, such as the hind limb, would have sufficiently high levels of TR to respond to T_3 early during metamorphosis compared with those transforming later, such as the tail, which is known to be the case (44). In addition, different direct T_3-response genes may function at different time points during transformation of a given tissue. For example, TRβ genes may function earlier, and its activation may be needed for the optimal expression of other T_3-response genes, such as TH/bZip, which may function later. By having TREs with different affinities at different genes, gene-specific temporal regulation of T_3-response genes can be achieved by regulating TR levels at least in part through autoinduction of the TRβ genes. Without such tissue- and gene-specific control of gene expression, some genes, such as TH/bZip, may be precociously activated. This would in turn lead to uncoordinated tissue transformations, thereby resulting in developmental defects.

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