Targeted chromatin binding and histone acetylation in vivo by thyroid hormone receptor during amphibian development

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Amphibian metamorphosis is marked by dramatic, thyroid hormone (TH)-induced changes involving gene regulation by TH receptor (TR). It has been postulated that TR-mediated gene regulation involves chromatin remodeling. In the absence of ligand, TR can repress gene expression by recruiting a histone deacetylase complex, whereas liganded TR recruits a histone acetylase complex for gene activation. Earlier studies have led us to propose a dual function model for TR during development. In premetamorphic tadpoles, unliganded TR represses transcription involving histone deacetylation. During metamorphosis, endogenous TH allows TR to activate gene expression through histone acetylation. Here using chromatin immunoprecipitation assay, we directly demonstrate TR binding to TH response genes constitutively in vivo in premetamorphic tadpoles. We further show that TH treatment leads to histone deacetylase release from TH response gene promoters. Interestingly, in whole animals, changes in histone acetylation show little correlation with the expression of TH response genes. On the other hand, in the intestine and tail, where TH response genes are known to be up-regulated more dramatically by TH than in most other organs, we demonstrate that TH treatment induces gene activation and histone H4 acetylation. These data argue for a role of histone acetylation in transcriptional regulation by TRs during amphibian development in some tissues, whereas in others changes in histone acetylation levels may play no or only a minor role, supporting the existence of important alternative mechanisms in gene regulation by TR.

Amphibian metamorphosis is a postembryonic developmental switch directly initiated by thyroid hormone (TH; refs. 1, 2). TH and in particular the biologically more active form, 3,5,3′-triiodothyronine (T₃), exerts its effects on target tissues via binding to TH receptors (TRs), which are transcription factors that belong to the nuclear receptor superfamily (3). TR modulates gene expression by binding to specific DNA sequences in target genes most often by forming a heterodimer with retinoid X receptors (RXRs) or 9-cis-retinoic acid receptors. TRs have the capacity to both activate transcription in presence of ligand and repress transcription in its absence. Recent evidence supports the hypothesis that TRs switch, in a ligand-dependent manner, between the binding of a corepressor complex (4) and the binding of a coactivator complex (5). The observations that transcriptional activation may be associated with the recruitment of histone acetyl transferase and repression by the recruitment of histone deacetylase has led to a model in which chromatin remodeling targeted by TRs contributes in part to transcriptional control (4–7). However, there is no evidence to date demonstrating whether such a mechanism of TR regulation of gene silencing and activation is involved in any physiological event. Amphibian metamorphosis provides an ideal model to address this aspect (2, 8). In Xenopus laevis, TRs are encoded by four genes (two TRα and two TRβ; ref. 9). TRβ genes are up-regulated at the transcriptional level during metamorphosis in response to endogenous TH synthesis (10–12). However, TRα genes are activated during late embryogenesis, well before the maturation of the thyroid gland and synthesis of endogenous TH (13). This expression pattern of TRα suggests a role for unliganded TR in gene silencing before TH-dependent gene activation during metamorphosis.

To investigate how TRs regulate gene expression during amphibian development, we examined in vivo TR binding and histone acetylation level on TH response gene promoters by using a chromatin immunoprecipitation assay with nuclei from whole embryos, tadpoles, or isolated tissues at various developmental stages. Our results indicate that TRs binds to TH response elements (TREs) in chromatin constitutively during development and that the modulation of histone acetylation is important for gene regulation by TRs.

Materials and Methods

Animals and Treatment. Adults and stage 55 premetamorphic tadpoles of the South African clawed frog Xenopus laevis were obtained from Nasco (Fort Atkinson, WI). Embryos were prepared by in vitro fertilization as described (14). Approximately 100 embryos at stage 20 and 20 tadpoles at stage 47 were treated for 1 day with 100 nM T₃ (Sigma), and/or 100 nM trichostatin A (TSA; Wako Biochemicals, Osaka), a specific histone deacetylase inhibitor (16). For analysis of gene regulation in the intestine, 12 stage 55 tadpoles were treated in 4 liters of dechlorinated tap water with or without 10 or 50 nM T₃ and/or 100 nM TSA for 2 days without feeding. The animals were then killed by decapitation, after anesthesia, for intestine and tail isolation.

RNAs Extraction and PCR Analysis of Gene Expression. RNAs were extracted from embryos, tadpoles, or isolated intestine or tail with RNAzol B (Tel-Test, Friendswood, TX) according to the manufacturer’s instructions. RNAs were resuspended in diethyl pyrocarbonate-treated water and quantified by UV absorption. Analyzing the RNAs on an agarose gel with ethidium bromide showed 18S and 28S rRNAs and the expected expression of gene of interest and the internal control gene, the ribosomal protein gene rpL8 (2 μM each) were mixed in 10 μl of total RNA in 20 μl as follows: RNAs and specific primers for the gene of interest and the internal control gene, the ribosomal protein gene rpL8 (2 μM each) were mixed in 10 μl of 50°C for 5 min, and allowed to cool down to room temperature. A
Preparation of Nuclei and Chromatin Immunoprecipitation (ChIP).
Nuclei were isolated as described (19). The pellet was resuspended in 360 μl of nucleus isolation buffer (0.25 M sucrose/10 mM Tris-HCl, pH 7.5/3 mM CaCl2/1 mM PMSF/1 μg/ml aprotinin/1 μg/ml pepstatin). Proteins were crosslinked to DNA by adding formaldehyde (37%) directly to nuclear resuspension to a final concentration of 1% and incubated for 30 min at room temperature. The nuclei were pelleted and then resuspended in 200 μl of lysis buffer (1% SDS/50 mM Tris-HCl, pH 8.1/10 mM EDTA/1 mM PMSF/1 μg/ml aprotinin/1 μg/ml pepstatin) for 10 min on ice. The lysate was sonicated 15 times with 15-sec pulses by using a sonicator set to 70% of maximum power to reduce DNA length to between 200 and 1,000 bp. At this step, it is essential to keep the samples cold all of the time. Debris was removed by centrifugation for 10 min at 14,000 × g at 4°C. The DNA was quantified and adjusted to equal concentration for the following chromatin solution. ChiP assay was done using a kit from Upstate Biotechnology. Chromatin solution (1 ml) was used for each ChiP assay with 5 μl of anti-acetylated histone H4 antisemur (Upstate Biotechnology), 8 μl of anti-Xenopus TR antisemur (recognizing both TRα and TRβ), anti-Xenopus RXRs antisemur (which recognizes RXRα but not RXRγ and has not been tested on RXRβ) (20), or anti-Xenopus Rpd3 antisemur (21). Chromatin solution (500 μl) was used for the control of input DNA in the chromatin solution. After the ChiP protocol, the recovered DNA was resuspended in 20 μl of H2O for the ChiP samples and 40 μl for the input control. Semiquanitative PCR was performed in 50 μl with 5 μl of 10 times Ex Taq buffer (Takara Shuzo), 8 μl of dNTP mix (2.5 mM each, Takara Shuzo), 2 μl of each primer (2 μM each, GibCO/BRL), 0.5 μl Ex Taq polymerase (5 units/μl, Takara Shuzo), 2 μl of the DNA sample, and 1 μl (1 μCi) of [α-32P]dTTP. The primers used for TRβ promoter: forward 5′-GTAAGCTGTAGCTGGATTAC-3′ and reverse 5′-GACAAGCTAGGAAGCTGGT-3′ (10); for TH/bZip promoter: forward 5′-TCTCTCTAATTCATCAAGGACTGTT-3′ and reverse 5′-CTTAAACCTCAGCTATTG-3′ (22); for a segment of TRβ transcribed sequence: forward 5′-CAGAAGACTGACACACTCAGATTCA-3′ and reverse 5′-CATTCTCCTCCCTCCCTCGGCGCATT-3′ (located respectively in exons 3 and 4; ref. 23); and for the intestinal fatty acid binding protein (IFABP) promoter:

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forward 5′-ATAGGCCTTTACGTGGAGGC-3′ and reverse 5′-GGCCACAAGATCTACTCG-3′ and reverse 5′-GACGACCAGTACGACGA-3′
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used to immunoprecipitate formaldehyde-crosslinked, sonicated chromatin fragments from nuclei isolated from embryos and tadpoles at different developmental stages. The TR or RXR-bound DNA fragments that were immunoprecipitated by the respective antibody were then analyzed by semiquantitative PCR by using primers flanking TH response elements in TH target genes.

For this purpose, we chose TRβA and TH/bZip genes, the only two Xenopus direct TH-response genes whose promoters have been characterized (10, 11, 22). The region selected for ChIP analysis contained the TRE (two direct repeats separated by 4 bp, 10, 11, 22) of the corresponding promoter but not any other nuclear receptor binding sites. As shown in Fig. 1B, ChIP analysis with antibodies to TR and RXR revealed little or no TR or RXR binding to the TRE fragment in either promoter in embryos at stage 20. The up-regulation of TRα in stage 47 tadpoles coincided with an increase in both TR and RXR binding to both promoters (Fig. 1B). As there is no detectable TH at this premetamorphic stage (15), these results indicate that unliganded receptor could bind to TREs in chromatin in vivo. We assume that this binding was most likely by TR/RXR heterodimers, as they are the preferred binding complexes for the TREs, although our assay could not distinguish the binding by TR/RXR heterodimers or that by TR/TR homodimers.

**Differential Effects of T3 and TSA on the Regulation and Histone Acetylation Levels of TH Response Gene Promoters During Early Development.** We next investigated whether the chromatin-bound TR/RXR regulate TH response genes in a T3- and histone acetylation-dependent manner. We treated embryos and tadpoles with T3 or histone deacetylase inhibitor TSA and then determined their effects on TRβ and TH/bZip gene regulation. At stage 20, neither T3 nor TSA was able to induce the expression of either T3 response gene (Fig. 2A). At stage 47, T3 treatment led to the activation of both TRβ and TH/bZip genes whereas TSA had no effect on either one (Fig. 2A). ChIP assay with TR antibody showed that the T3 or TSA treatment had no effect on TR binding to the TREs of TRβ and TH/bZip. Thus, TR is bound to the TREs constitutively in premetamorphic (stage 47) tadpoles but not in embryos at stage 20 (Fig. 2B), consistent with the very low level of TRs in embryos (Fig. 2A). ChIP assay with RXR antibody showed that like TR, RXR also was constitutively bound to TREs in stage 47 tadpoles (Fig. 1 and data not shown). Thus, TR/RXR binding to the TREs enables the TH response genes to respond to T3 treatment, whereas blocking histone deacetylase activity has little effect on the overall expression of the genes in the animals.

To investigate whether T3 or TSA altered the status of histone acetylation on the chromatin of the promoter regions, we analyzed histone acetylation levels by ChIP assay. Antibodies specific to acetylated histone H4 were used to immunoprecipitate formaldehyde-crosslinked sonicated chromatin from nuclei isolated from stage 20 embryos and stage 47 tadpoles. The results showed that T3 alone had no effect on histone acetylation levels of either promoter in both the embryos and tadpoles (Fig. 2C). On the other hand, TSA caused remarkable increases in the acetylation levels on the promoters in both embryos and tadpoles (Fig. 2C). Interestingly, the acetylation levels at both TRβ and TH/bZip promoters were higher in premetamorphic tadpoles (stage 47), when TRα expression was high, than in embryos (stage 20), when TRα expression was very weak (Fig. 2C). Thus, the binding by unliganded TR/RXR keeps the genes repressed despite the increase acetylation levels as the animal develops. On the other hand, increasing histone acetylation alone, by TSA treatment, is insufficient to alter the expression levels of TH-response genes in whole animals.

**T3 Causes the Release of the Histone Deacetylase Rpd3 from TH-Response Genes.** Histone acetylation levels are determined by the action of histone acetylases and deacetylases. Unliganded, but not T3-bound, TR is known to interact with corepressor complexes containing histone deacetylases. Even though histone acetylation levels were found to be unchanged by T3 when analyzed in whole animals (Fig. 2C), it is possible that T3 treatment of premetamorphic tadpoles can influence the recruitment of histone acetylases/deacetylases to specific promoters. Thus, we next examined in tadpoles at stage 47 whether unliganded TR recruited histone deacetylases and whether T3 induced the release of histone deacetylases. ChIP assay with polyclonal antibody to histone deacetylase Rpd3, the only characterized Xenopus histone deacetylase (21), revealed that Rpd3
Changes in Histone Acetylation Levels Correlate with Gene Regulation by TR During Intestine Metamorphosis. To further investigate the role of histone acetylation in regulation of the TH response genes, we examined whether T₃ or TSA treatment altered the levels of histone H₄ acetylation on TRβ- and TH/bZip-promoter chromatin. First, nuclei were isolated from intestine, tail, and whole tadpole at stage 55 after treatment with T₃ or TSA and used for ChIP assay with an antibody specific to acetylated histone H₄. As observed for stage 47, in whole tadpoles stage 55, the level of the acetylation levels on histone H₄ in TRβ- and TH/bZip-promoter chromatin did not change (Fig. 5A). However, in the intestine and tail, T₃ treatment led to an increase in the acetylation levels on histone H₄ in chromatin of both promoters (Fig. 5A), as did TSA except for TH/bZIP in the tail (Fig. 5A). Second, by using an antibody against acetylated lysine, Western blotting of proteins isolated from nuclei of stage 55 tadpole intestine treated with the histone deacetylase inhibitor revealed that TSA-induced hyperacetylation of histones (Fig. 5B). T₃ treatment caused little change in overall core histone acetylation (Fig. 5B). This may not be surprising because T₃ can both activate and repress, directly or indirectly, gene expression in various target tissues, and it would be expected to alter the histone acetylation levels of only its target genes but not globally. As a control, ChIP analysis of a transcribed region of TRβ gene between exons 3 and 4, which is >40 kb away from the TRβ promoter (9, 23), revealed that histone H₄ acetylation level was very low and more importantly, not affected by either T₃ or TSA treatment (Fig. 5C). Finally, as another control for specificity of the induced local histone hyperacetylation, we analyzed the acetylation level of IFABP promoter. The IFABP gene is
H4 acetylation levels at the promoter of IFABP gene, which is not a directly T3 treatment has no effects on histone H4 acetylation in the transcribed region of 13142. Consistently, we found that IFABP was not expressed in the tail expressed only in the intestinal epithelial cells (29). It is not a T3 direct response gene and its promoter lacks a TRE (24, 29). Consistently, we found that IFABP was not expressed in the tail but was expressed in the intestine (ref. 29 and data not shown). ChIP assay showed that the chromatin of its promoter contained acetylated histone H4 in the intestine but not in the tail (Fig. 5D). Furthermore, in the intestine, the level of histone H4 acetylation was not affected after a T3 treatment but slightly increased after TSA treatment (Fig. 5D). These results indicate that the observed change in histone acetylation levels is restricted to T3 target genes in specific tissues and is not a non-specific effect of the hormone treatment. Taken together, these data suggest that T3-induced gene expression requires histone hyperacetylation of chromatin at/near the TREs of target genes in vivo.

Discussion

In vitro biochemical and tissue culture transfection studies have shown that TR/RXR heterodimers can repress or activate gene transcription depending upon the absence or presence of TH. Our earlier work using the frog oocyte system indicates that such dual functions of the receptors persist even in the context of chromatin (12, 30–32, 42). Recent findings by us and others suggest that unliganded TR can recruit histone deacetylases, whereas T3-bound TR can recruit histone acetyltransferases (acetylases) to target genes (5, 7, 33, 42). This result has strengthened the connection between histone acetylation, chromatin remodeling, and hormone-induced gene regulation. However, there is a lack of in vivo evidence demonstrating the dual functions of TR/RXR and the physiological consequence of changes in histone acetylation as a mechanism in TR-mediated transcriptional regulation. Our studies here provide a critical link between in vitro studies and developmental/physiological roles of the receptors. Our major conclusions are (i) TR and RXR binds to TREs in chromatin constitutively during postembryonic development; (ii) in the absence of T3, histone deacetylase is present at TH response gene promoters, and (iii) changes in histone acetylation levels correlate with T3-dependent gene regulation in some but not all tissues.

Amphibian metamorphosis, a postembryonic process controlled by TH, is a unique model to study TR function in vivo during development. Before stage 35 (hatching stage) for Xenopus laevis, embryos are incapable of responding to exogenous TH. The lack of competence to respond to T3 has been suggested to be attributable to the lack of adequate levels of TRs (25). TRα genes are activated only after tadpole hatching (stage 35) whereas TRβ genes are repressed until metamorphosis (13, 20). We have demonstrated here for the first time in vivo that there is little TR binding in a chromatin context to TREs of TH response genes in embryos. Moreover, the increase in TR binding to TREs as the embryos develop into tadpoles (e.g., from stage 20 to 47) associated with the up-regulation of TRα expression is directly correlated with the ability of TH response genes to be activated by T3 treatment in premetamorphic tadpoles. Our results further show that both TR and RXR are bound to the TREs in chromatin, although our assay does not allow us to distinguish between the binding of TR/TR homodimers and TR/RXR heterodimers to TREs. On the other hand, these results together with our earlier studies showing that over-expression of TRs and RXRs together but not individually in Xenopus embryos leads to specific regulation of TH response genes (14) provide a molecular basis, that is, the expression of TR and RXR genes, for tadpole competence (i.e., the ability to respond physiologically to T3).

TR/RXR appears to have dual functions, repressing TH response genes in premetamorphic tadpoles but activating them during metamorphosis. How TR represses genes in vivo during development is yet unclear. Here we have demonstrated that (i) T3 treatment lead to the release of histone deacetylase Rpd3 from the TRE regions of TH response genes in premetamorphic tadpoles, and (ii) treatment with the histone deacetylase inhibitor, TSA, leads to the activation of T3 response genes in vivo in...
the intestine and tail of premetamorphic tadpoles. These results suggest that during premetamorphic development, unliganded TRs are likely to repress transcription, in part by recruiting corepressor complexes containing histone deacetylases, although direct experimental proof remains to be obtained.

The dynamics of histone acetylation provides an attractive mechanism for the reversible activation and repression of transcription (7, 35). Liganded TRs can recruit coactivator complex. Diverse coactivator proteins have been shown to have histone acetyltransferase activities (6, 36). Our in vivo data shows that T3 treatment leads to the release of histone deacetylase at T3 response gene promoters. We also show at least in the intestine and the tail, that T3 increases local histone H4 acetylation, which may be contributed by the release of deacetylase complexes and/or concurrent recruitment of acetylase complexes. These findings are consistent with the idea that histone acetylation is associated with transcriptionally competent chromatin and hypoacetylated histones with transcriptionally silent chromatin, and they support a role for alterations of histone acetylation levels alone is insufficient to change gene expression. Although this model is likely an over-simplified one considering our current knowledge on the involvement of other cofactor complexes that do not alter histone acetylation levels and the ability of non-histone transcription factors and cofactor to be acetylated (40, 41), such a mechanism for the reversible activation and repression of transcription factors and cofactor to be acetylated (40, 41), such a mechanism for the reversible activation and repression of transcription factors and cofactor to be acetylated (40, 41), such a

In conclusion, our results show that in vivo during amphibian development, TRs are bound to TREs assembled into chromatin, whereas in the absence of hormone, they recruit histone deacetylase complexes to silence transcription in a tissue specific manner. Upon TH synthesis during metamorphosis, the receptors undergo conformational change that could lead to the release of deacetylase complexes and possible recruitment of acetylase complexes, resulting in increased histone acetylation and gene activation. Although this model is likely an over-simplified one considering our current knowledge on the involvement of other cofactor complexes that do not alter histone acetylation levels and the ability of non-histone transcription factors and cofactor to be acetylated (40, 41), such a

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