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RNA polymerase II phosphorylation at serine 2 and histone H3 tri-methylation at lysine 36 are key steps for thyroid hormone receptor β gene activation by thyroid hormone in *Rana catesbeiana* tadpole liver

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ABSTRACT

Thyroid hormone (TH) is essential for amphibian metamorphosis, during which the expression of many genes is controlled directly or indirectly through TH receptors (TRs). Thyroid hormone binding to TRs induces coregulator switching on regulatory regions of TH-inducible genes: corepressors complexed with unliganded TRs are replaced by coactivators complexed with liganded TR resulting in transcriptionally active states. The coregulator switching is linked to histone acetylation. In our study, we have investigated the acetylation and methylation states of histones H3 and H4 using chromatin immunoprecipitation (ChIP) assays on the 5′ coding region of the TRβ gene, a primary TH-response gene, in the liver from *Rana catesbeiana* tadpoles either treated with or not treated with 3,3′,5-triiodothyronine (T3). 3,3′,5-Triiodothyronine treatment for 3 days increased the amount of TRβ transcript by 19-fold. This increase was associated with increases in the acetylation of histone H4 and lysine 9 in histone H3 (H3-K9), and trimethylation of lysine 36 in histone H3 (H3-K36). In addition, the amounts of RNA polymerase II (PolII) and serine 2 phosphorylation in PolII (PolII-S2) increased. These results suggest that T3 treatment enhances the elongation activity of PolII on the TRβ gene in the liver by increasing histone H3-K36 trimethylation through PolII-S2 phosphorylation.

1. Introduction

Amphibian metamorphosis is obligatorily controlled by thyroid hormone (TH). Thyroid hormone levels in amphibian plasma gradually increase during the prometamorphic stages, peak in an early metamorphic climax stage, then decrease during the late metamorphic climax stages. The increase in TH levels induces morphogenetic changes, including the differentiation and proliferation of adult tissues and degeneration of larval-specific tissues by apoptosis, by binding to nuclear TH receptors (TRs) in a spatiotemporal manner [1]. In the liver, it has been thought that reprogramming of the gene expression profiles occurs without dramatic morphological changes during metamorphosis [2]. Thyroid hormone receptors are members of a multigene superfamily of nuclear receptors. The TR isoforms α and β are found in most vertebrates. Thyroid hormone receptors act as TH-dependent transcription factors that bind as heterodimers with 9-*cis* retinoic acid receptor isoforms to specific *cis*-acting DNA sequences, known as TH-response elements (TREs) on target genes [3].

Thyroid hormone receptors are proposed to have dual functional roles in amphibian metamorphosis: (1) unliganded TRs repress the transcription of TH-inducible genes by recruiting corepressors and promoting histone deacetylation before prometamorphic stages, whereas (2) TH-bound TRs activate the transcription of TH-inducible genes by recruiting coactivators and promoting histone acetylation during the prometamorphic and metamorphic climax stages [4]. However, this TH-dependent functional change of TRs does not occur at the same time in all tissues of metamorphosing tadpoles [5]. What determines the tissue-specific timing of TH-induced biochemical and morphological changes has not been fully elucidated; nevertheless, several factors have been postulated in model animals. Possible candidate factors include TH-transporters [6], deiodinases, cytoplasmic TH-binding proteins, receptors [5], coactivators/corepressors, and other chromatin-modifiers [7,8].

The regulation of TH-dependent genes involves histone modifications such as acetylation and methylation, which influences the recruitment of coactivators or corepressors to produce active or inactive complexes with TRs [9]. According to the histone code hypothesis [10,11], a specific combination of these modifications on the histone tails affects the chromatin structure in a regulatory

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region of a target gene and influences the recruitment of specific coactivators and corepressors and other chromatin-modifiers [9,12]. In the promoter vicinity of the *Xenopus laevis* TRβ gene in TH-responsive tissues or cells, repression of transcription by unliganded TRs was associated with decreases in the levels of acetylation of histones H3 and H4 [7,9,13] and methylation of histone H3 at lysine 4 (H3-K4), and an increase in the level of methylation of histone H3 at lysine 9 (H3-K9) [9]. Most of these modification states were reversed by the addition of TH [7,9,13]. Such changes in histone modification states are thought to influence RNA polymerase II (PolII) activity [14,15]. However, little is known about the various effects of histone modifications on gene transcription and how the TH signal is transferred to PolII on actively transcribing target genes.

To clarify the relationship between histone modifications and PolII activity on the primary TH-responsive gene, TRβ gene, we investigated histone acetylation and methylation and PolII phosphorylation in the liver of *Rana catesbeiana* either treated with or not treated with the TH 3,3′,5-triiodothyronine (T3) using chromatin immunoprecipitation (ChIP) assays. Here, we demonstrated that T3 increased the acetylation of histone H3-K9 and histone H4, and tri-methylation of lysine 36 in histone H3 (H3-K36). These changes were associated with an increase in the amount of PolII and serine 2 phosphorylation of PolII (PolII-S2) but not serine 5 phosphorylation of PolII (PolII-S5) on the TRβ gene.

2. Materials and methods

2.1. Animals and treatment

Premetamorphic *Rana catesbeiana* tadpoles at TK stage X–XI [16] were obtained from Saitama Amphibian Institute, Saitama, Japan. The tadpoles were maintained in aerated and dechlorinated tap water at 28 °C and fed boiled spinach three times a week. After acclimatization for 7 days, tadpoles (six/group) were treated with or without 5 nM 3,3′,5-triiodothyronine (T3) (Sigma–Aldrich, St. Louis, MI, USA) in 4 L of dechlorinated tap water for 3 days. Tadpoles were not fed during the 3-day treatment period. The tadpoles were then anesthetized with 0.02% 3-aminobenzoic acid ethyl ester (Sigma–Aldrich) and body weight, body length, tail length, and tail height were measured, and the liver dissected, in accordance with the code of ethics on the Animal Welfare Committee of Shizuoka University.

2.2. RNA extraction and PCR analysis of gene expression

Total RNA was extracted from the liver samples using the acidified guanidine thiocyanate method [17]. RNA was resuspended in diethylpyrocarbonate-treated water and quantified by UV absorption. Samples of RNA (1 μg) were treated with reverse transcriptase (SuperScript™ III RT, Invitrogen, Tokyo, Japan) according to the manufacturer's instructions and specific RNA was quantified by real-time PCR using the LightCycler 480 SYBR Green I Master (Roche Molecular Biochemicals, Tokyo) and a Light-Cycler instrument (Roche). The thermocycler program included denaturation at 95 °C (5 min) then 50 cycles of 95 °C (10 s), 60 °C (10 s), and 72 °C (6 s). The endpoint used in real-time PCR quantification, Ct, was defined as the PCR cycle number that crossed an arbitrarily placed signal threshold and is a function of the amount of target DNA present in the starting material. Amounts were quantified using the formula 2^{-Ct}. To standardize each experiment, the amount of TR transcript was divided by the amount of β-actin transcript. Primer sequences used were as follows: TRβ transcript (accession number: L27344): sense 5′-caagggctttttcaggagaac-3′ and antisense 5′-ttggcactggttccttgttac-3′; TRα transcript (accession number: L06064): sense 5′-gaagcgagaacagctgaagaa-3′ and antisense 5′-acttccgtgtcatccagattg-3′; and β-actin transcript (accession number: AB094353): sense 5′-gaaagagaaatcgtgcgtgac-3′ and antisense 5′-gtttccgatggtgatgacttg-3′.

2.3. Preparation of nuclei and ChIP

Liver samples (~0.5 g) were homogenized in 10 mL of fixation solution (1% formaldehyde, 4.5 mM HEPES, pH 8.0, 9 mM NaCl, 0.09 mM EDTA, and 0.04 mM EGTA) and incubated for 30 min at 37 °C to crosslink proteins with DNA [18]. After 30 min, 1 mL of 1.5 M glycine was added to each liver sample to stop the reaction. Each sample was then washed with FACS solution (1 × PBS, 2% bovine serum and 0.05% sodium azide) and centrifuged briefly (15 min at 1600*g*) at 4 °C twice. The pelleted sample was vortexed in 1 mL of SDS lysis buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1% SDS) then incubated for 2 h on ice to solubilize the cells. The lysate was sonicated (12 × 30-s pulses; Sonifier, Branson Ultrasonics Corp., CT, USA) to obtain chromatin fragments between 200 and 500 base pairs. Debris was removed by centrifugation for 15 min at 20,000*g* at 6–8 °C. The protein concentration of the supernatant was quantified by the Lowry method [19] and the concentration of the supernatant was adjusted to 3.24 mg/mL to produce a chromatin solution. Chromatin solution (23 μL or 28 μL) was used for each ChIP assay [20] with 0.6 μg of antibody. Antibodies used were anti-acetylated histone H4 antibody (Millipore, Tokyo, Japan), anti-acetylayted histone H3-K9 antibody (Millipore), anti-mono-, anti-di-, and anti-tri-methylated histone H3-K4 antibodies (Abcam Japan, Tokyo, Japan), anti-mono-, anti-di-, and anti -tri-methylated histone H3-K9 antibodies (Millipore), anti-mono-, anti-di-, and anti-tri-methylated histone H3-K36 antibodies (Abcam Japan), anti-PolII antibody (Covance, Berkeley, CA, USA), anti-phosphorylated PolII-S2 and PolII-S5 antibodies (Abcam Japan), or normal rabbit IgG (Sigma). Real-time PCR was done using the following primers for the TRβ coding region +100 to +300: forward 5′-caagggctttttcag-

Table 1
The effect of T3 treatment on the morphology of and transcriptional activation of hepatic thyroid hormone receptor (TR) genes in premetamorphic *Rana catesbeiana* tadpoles.

	Day 0		Day 3	
	Control	T3-treated	Control	T3-treated
Body weight (g)	5.9 ± 1.3	4.6 ± 0.7	5.5 ± 1.4	3.6 ± 0.6
Body length (cm)	8.4 ± 0.6	7.8 ± 0.5	8.3 ± 0.6	7.3 ± 0.4
Tail length (cm)	5.1 ± 0.3	4.7 ± 0.2	4.7 ± 0.3	4.1 ± 0.3
Tail height (cm)	1.7 ± 0.2	1.4 ± 0.1	1.6 ± 0.2	1.0 ± 0.1*
Tail length/Body length	0.61 ± 002	0.61 ± 0.02	0.56 ± 0.02	0.55 ± 0.01
Tail height/Body length	0.20 ± 0.01	0.18 ± 0.00	0.19 ± 0.01	0.13 ± 0.01**,##
TRα induction (fold)	–	–	1.0 ± 0.3	1.6 ± 0.5
TRβ induction (fold)	–	–	1.0 ± 0.4	19.1 ± 4.7**

Tadpoles (n = 6/each group) were reared in 4 L of a dechlorinated tap water in the presence or absence of 5 nM T3 for 3 days. Data are mean ± SEM. *P < 0.05 and **P < 0.01 compared with control (T3-untreated) on day 3; and ##P < 0.01, compared with T3-treatment on day 0.