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ABSTRACT

To elucidate molecular mechanisms by which the phenolic herbicide ioxynil (IOX) and the brominated flame retardant tetrabromobisphenol A (TBBPA) exert thyroid hormone (TH)-disrupting activity, we investigated the effects of the chemicals on the histone and RNA polymerase II (RNAPII) modifications in *Xenopus laevis* XL58-TRE-Luc cells in direct TH-response genes encoding TH receptor β (Thrb) and TH-induced basic leucine zipper transcription factor (Thibz) using chromatin immunoprecipitation (ChIP) assays. For both the *thrb* and *thibz* genes, 3,3′,5-triiodothyronine (T3) enhanced the amounts of gene transcripts and increased the amounts of acetylated histone H4 (H4Ac), trimethylated histone H3 lysine 4 (H3K4me3) and phosphorylated RNAPII serine 5 (RNAPIIS5P), epigenetic markers of gene activation at 5′ regulatory regions, and the amounts of trimethylated histone H3 lysine 36 (H3K36me3) and phosphorylated RNAPII serine 2 (RNAPIIS2P), epigenetic markers of activation of transcriptional elongation at protein-coding regions. Treatment with IOX and TBBPA reduced the amounts of the *thrb* transcript and suppressed the T3-induced modifications of H3K4me3, RNAPIIS5P, H3K36me3 and RNAPIIS2P. In the *thibz* gene, IOX and TBBAP did not suppress the T3-induced the histone and RNAPII modifications except for H3K36me3 in the TBBPA treatment, despite both chemicals decreasing the T3-induced transcription. Our results demonstrate that IOX and TBBPA affect TH-induced histone and RNAPII modifications, which are involved in early and progressive stages of RNAPII transcriptional elongation, in direct TH-response genes, in somewhat target gene-dependent and chemical-specific manners. Both IOX and TBBPA are likely to influence epigenetically a cascade of TH receptor-mediated gene regulation.

Key Words: epigenetics; histone; ioxynil; RNA polymerase II; tetrabromobisphenol A; thyroid hormone receptor.

INTRODUCTION

Chemicals that interfere with the thyroid system target various sites of the hypothamalic-pituitary-thyroid axis, including the production and release of thyroid hormone (THs) from the thyroid gland, the distribution of THs in blood, the uptake of THs by target tissues, metabolic enzymes that activate or inactivate THs, and TH receptors (Boas *et al*., 2012). Some of these chemicals share structural resemblance to THs. TH distributor proteins in plasma, metabolic enzymes and TH receptors have significant affinity for such chemicals, e.g., polychlorinated biphenyls or their hydroxyl forms, polybrominated flame retardants and halogenated phenols (Ulbrich and Stahlmann, 2004; Hamers *et al*., 2006; Boas *et al*., 2012).

THs play important roles in growth, development, differentiation, basal metabolic rate and protein synthesis in vertebrates. In amphibians, 3,3′,5-triiodo-L-thyronine (T3) induces a cascade of tissue-specific gene regulation responsible for metamorphosis through TH receptors, members of nuclear receptor superfamily. Therefore, amphibians have been used as a model system for elucidating action mechanisms and environmental assessments of thyroid disrupting chemicals (OECD, 2004). The TH receptor β (*thrb*) gene, one of early direct TH-response genes, is autoregulated by binding liganded TH receptor complexes to TH-response elements (TREs) in regulatory regions at the onset of metamorphosis (Tata *et al*., 1993). TH-induced basic leucine zipper transcription factor gene (*thibz*) is another example of direct TH-response genes in *Xenopus laevis*, with biphasic kinetics that are intermediate between early direct and late indirect TH-response genes (Furlow and Brown, 1999). This biphasic kinetics may be due to lower affinity binding of TH receptors to TREs on the *thibz* gene (Buchholz *et al*., 2005) or the presence of potential sites for many transcription factors other than TREs (Furlow and Brown, 1999). Activation of these genes is progressed with subsequent recruitment of coactivator protein complex (Buchholz *et al*., 2006), resulting in epigenetic changes including histone modifications such as acetylation (Sachs and Shi, 2000; Havis *et al*.,

2003; Bilesimo *et al*., 2011; Grimaldi *et a*l., 2013).

Ioxynil (IOX), an iodine-containing phenolic herbicide, which was widely used for selective post-emergence weed control, targets photosystem II in plant chloroplasts (Takahashi *et al*., 2010). It has been used at ~80 tones/ year in Japan (METI, 2011). Near agricultural areas, IOX levels were reported as high as 0.9 μg/L in some water systems (Helweg, 1994). IOX is characterized by low water solubility and a tendency to bioaccumulate, and known to exert carcinogenicity and reproductive toxicity with an acute LC_{50} at 3.5–8.5 mg/L in fish (European Commission, 2004). Tetrabromobisphenol A (TBBPA), a brominated derivative of bisphenol A, is one of the most commonly used polybrominated flame retardants. It is used in electronic equipments, paints, plastics, and synthetic textiles. The annual production of TBBPA has been estimated to be over 210,000 tons/year (Alaee *et al*., 2003). Although more than 90% of TBBPA administrated was excreted into feces within 72 h in rats (Kuester *et al*., 2007), TBBPA has been detected in various biological samples including food, maternal breast milk (Shi *et al*., 2009).

IOX and TBBPA are known to interfere with the thyroid system. In previous studies, IOX and TBBPA suppressed the transcriptional activation of TH-response genes in an amphibian cell culture system in a similar fashion (Sugiyama *et al*., 2005b; Kudo *et al*., 2006). However, IOX and TBBPA may not suppress the transcriptional activation of TH-response genes by the same mechanism. At the same concentrations (10^{-7} to 10^{-6} M), IOX did not compete with T3 for binding to amphibian TH receptors in vitro (Yamauchi *et al*., 2002; Ishihara *et al*., 2003; Shimada and Yamauchi, 2004), whereas TBBPA did (Kudo *et al*., 2006). In contrast to IOX, TBBPA showed a weak agonist activity in the absence of T3 in a TH-dependent luciferase assay (Kudo *et al*, 2006), indicating that the direct interaction of TBBPA with TH receptors in vivo is highly likely.

To determine if differences exist in the molecular mechanisms of how IOX and TBBPA suppress the transcriptional activation of TH-response genes, we investigated the effects of IOX and TBBPA on the TH-induced transcriptional activation of the *thrb* and *thibz* genes. To do this, we measured the effect of

IOX and TBBPA on transcript abundance of the T3-induced *thrb* and *thibz* genes using reverse transcription (RT) real-time quantitative polymerase chain reaction (qPCR) and then measured the level of epigenetic modifications of histones, i.e., acetylation and methylation, and RNA polymerase II (RNAPII), i.e., phosphorylation, in the regulatory and protein coding regions of the *thrb* and *thibz* genes using chromatin immunoprecipitation (ChIP).

MATERIALS AND METHODS

Reagents and antibodies. T3 (>98% purity) was obtained from Sigma (St. Louis, MO), and IOX (3,5-diiodo-4-hydroxybenzonitril, analytical standard; >99% purity) was purchased from Wako Pure Chemical Industries (Osaka, Japan). TBBPA (>98% purity) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Antibodies against trimethylated histone H3 lysine 4 (H3K4me3), trimethylated histone H3 lysine 36 (H3K36me3), phosphorylated RNAPII serine 2 (RNAPIIS2P) and phosphorylated RNAPII serine 5 (RNAPIIS5P) were obtained from Abcam (Tokyo, Japan). Antibodies against acetylated histone H4 (recognizes acetylated lysines 5, 8, 12 and 16; Cat. No. 06-866) (H4Ac) and RNAPII, and normal rabbit IgG were purchased from Millipore (Tokyo, Japan), Covance (Berkeley, CA, USA), and Sigma, respectively.

All other chemicals used in this study were of the highest grade available and were purchased from Wako Pure Chemical Industries or Nacalai Tesque (Kyoto, Japan). As stock solutions, T3 was dissolved in dimethylsulfoxide to a concentration of 5 mM, and IOX and TBBPA were dissolved in dimethylsulfoxide to a concentration of 30 mM. Control assays without IOX or TBBPA were performed in the presence of dimethylsulfoxide alone at the same concentration (final 0.2%).

Cell culture and luciferase reporter analysis. Recombinant *X. laevis* XL58-TRE-Luc cells were used

for luciferase assay. This cell line was established by introducing the firefly *luciferase* gene under the control of SV40 promoter and the *X. laevis* TREs derived from the *thibz* promoter and the *enhanced green fluorescent protein* gene under the control of CMV promoter (Sugiyama *et al*., 2005a), and expressed luciferase activity in a T_3-dependent manner (Sugiyama *et al*., 2005a). The cells (2×10^5 cells/well) were pre-incubated for 18 h at 25°C in 24-well culture plates (Nunc, Roskilde, Denmark) in 70% Leibovitz's L-15 medium containing 10% fetal bovine serum stripped with AG-1X-8 resin (Bio-Rad, Hercules, CA, USA) to remove T3 (Samuels *et al*., 1979). Cells were then cultured in serum-free 70% Leibovitz's L-15 medium with or without 2 nM T_3 and in the absence or presence of 0.01–1.0 μM test chemicals for 24 h. Cells were lysed with 100 μL of Passive Lysis Buffer (Promega, Madison, WI, USA) for 15 min. The cell lysate was immediately assayed for firefly luciferase activity using the Bright-Glo Luciferase Assay System (Promega).

RNA extraction and RT-qPCR analysis. After treatment with or without 2 nM T3 and in the absence or presence of 1 μM test chemicals for 24 h, XL58-TRE-Luc cells (2×10^6 cells/90 mm dish) were harvested and lysed with 500 μL of the acid guanidinium thiocyanate solution (Chomczynski and Sacchi, 1987). The total RNA was isolated with phenol and chloroform. To confirm its integrity, RNA (1 μg per lane) was electrophoresed in a 1% agarose gel containing 2.6 M formaldehyde, and 28S and 18S rRNAs were visualized by ethidium bromide staining. Complementary DNAs were synthesized from the total RNA (200 ng) in 10 μL of 1× Taqman RT buffer using Taqman RT reagents kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Amounts of specific transcripts were estimated using Power SYBR Green Master Mix and ABI Prism 7000 sequence detection System (Applied Biosystems) with a specific primer set (each 400 nM) (Supplementary table S1 and Fig. 1). Each PCR was run in triplicate to control for PCR variation. The thermocycler program included a step of denaturation at 95°C (10 min), and 40 cycles of 95°C (15 s), 60°C (1 min), and 50°C (2 min). The endpoint used in

real-time PCR quantification, C_q, was defined as the PCR cycle number that crosses an arbitrarily placed signal threshold and is a function of the amount of target DNA present in the starting material. Quantification was determined by applying the 2^{-Cq} formula and calculating the average of the three values obtained for each sample. Eligibility of this formula was verified using a mixture of *X. laevis* cDNAs containing a test cDNA at five different concentrations (1:50:250). To standardize each experiment, the amounts of the *thrb* and *thibz* transcripts were divided by those of ribosomal protein L8 (*rpl8*) transcript in the same sample by the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). Detailed information about RT-qPCR is shown in Supplementary tables S1 and S2.

ChIP assay. ChIP assay was performed as previously described (Mochizuki *et al*., 2012) with some modifications. Briefly, chromatin in cultured cells (2×10^6 cells/90 mm dish) was cross-linked in 7.7 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) at 25°C. After 15 min, 1.5 M glycine was added up to one-tenth the volume of the fixation solution to stop the cross-linking. Cells were collected from a culture dish using a rubber policeman, and were then washed with 10 mL of FACS solution (1× phosphate buffered saline, 2% bovine serum and 0.05% sodium azide) by centrifugation at 1,500 × g for 15 min at 4°C twice. The pelleted sample was vortexed in 1 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulfate) then incubated for 1 h on ice to solubilize the cells. The lysate was sonicated (12 × 30-s pulses; Ultra5 homogenizer, VP-55, TAITEC, Saitama, Japan) to obtain DNA fragments between 200 and 500 base pairs. Debris was removed by centrifugation at 18,000 × g for 15 min at 7°C. The chromatin sample was diluted ten-fold with ChIP dilution buffer (50 mM Tris-HCl, pH 8.0, 167 mM NaCl, 1.1% Triton X-100, 0.11% sodium deoxycholate). The diluted sample (1 mL) was mixed with 50% Protein G Sepharose slurry (10 µL) containing 100 µg/ml salmon sperm DNA and 1% BSA, and then incubated in a rotator for 4 h at 4°C. The supernatant was used for input (50 µL) and each ChIP assay (225 µL). The amounts of the supernatant were empirically

determined to obtain the data comparable to previous ones (Mochizuki et al. 2012). Chromatin solution was incubated in a rotator at 4°C overnight with each antibody (0.3-0.6 μg depending antibodies used) or normal IgG. The 50% Protein G Sepharose/salmon sperm DNA/BSA slurry (5 μL) was added to the chromatin-antibody solution and then this solution was incubated for 18 h at 4°C. The immunoreactive chromatin was recovered in 400 μL of ChIP direct elution buffer (10 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5 mM EDTA and 0.5% sodium dodecyl sulfate). After reverse cross-linking by heating the samples at 65°C overnight and treating with 50 μg/mL Proteinase K and 10 μg/mL ribonuclease A, one twenty-fifth of the extracted DNA was subjected to qPCR using primer sets corresponding to the indicated sites in the promoter and coding regions. Amounts of DNA were quantified using the formula 2^{-Cq}. The C_q values of the ChIP signals were expressed as percentages of the ChIP signals for the input DNA. Detailed information about qPCR is shown in Supplementary tables S1 and S2.

Statistical analysis. All assay data are presented as mean ± standard error of the mean (SEM). Differences between groups were analyzed by one-way analysis of variance using the Fisher's least significant difference test for multiple comparisons to show significant differences. $p < 0.05$ was considered statistically significant.

RESULTS

Effects of IOX and TBBPA on TH-Dependent Luciferase Activity and Expression of Direct TH-Response Genes

Both IOX and TBBPA inhibited the T3-induced luciferase activity in XL58-TRE-Luc cells in a concentration-dependent manner (Fig. 2A), in agreement with the previous reports (Sugiyama *et al*., 2005b; Kudo *et al*., 2006). In XL58-TRE-Luc cells, 2 nM T3 increased the luciferase activity 2.1- to 2.6-fold. The

addition of 1 μM IOX inhibited the T3-induced luciferase activity by 45 ± 3 %, whereas the addition of 1 μM TBBPA inhibited the T3-induced luciferase activity by 29 ± 3 %. The inhibition effect of the chemicals was abolished when the cells were treated with the chemicals at the same concentration for the last 0.5 h (data not shown), suggesting that IOX and TBBPA may not interfere directly with the enzyme activity of luciferase.

IOX and TBBPA also suppressed the T3-induced transcript levels of the *thrb* and *thibz* genes, in agreement with the previous report for IOX (Sugiyama *et al*., 2005b). Treatment with T3 increased the amount of the *thrb* transcript 7-fold and the *thibz* transcript 3-fold (Fig. 2B). The addition of 1 μM IOX or TBBPA suppressed the T3-induced transcript *thrb* levels by 43% to 44%, and suppressed the T3-induced transcript *thibz* levels by 55% (IOX) and 32% (TBBPA) (Fig. 2B).

Effects of IOX and TBBPA on Histone H4 Acetylation and Recruitment of RNAPII in Direct TH-Response Genes

Both IOX and TBBPA had no effect on the amounts of H4Ac and RNAPII in the 5′ regulatory regions of the *thrb* and *thibz* genes (panels *I* and *V* in Figs. 3A and 3B). T3 increased the amounts of H4Ac and RNAPII in the 5′ regulatory regions of the *thrb* and *thibz* genes; however, the addition of IOX and TBBPA did not increase or decrease the amounts of H4Ac and RNAPII. The amounts of H4Ac and RNAPII in the coding regions of the *thrb* and *thibz* genes were significantly lower than that in the 5′ regulatory regions (panels *II–IV* versus panel *I* and panels *VI–VIII* versus panel *V* in Figs. 3A and 3B), with no significant changes among four treated or untreated groups. The ChIP signals for normal rabbit IgG were ≤0.2% of input in both genes and were not significantly changed among the groups (data not shown).

Effects of IOX and TBBPA on Trimethylation of Histone H3K4 and Phosphorylation of RNAPIIS5 in Direct TH-Response Genes

IOX and TBBPA suppressed the amounts of T3-induced H3K4me3 and RNAPIIS5 in the 5′ regulatory region of the *thrb* gene (panels *I* and *V* in Fig. 4A), but not in the *thibz* gene (panels *I* and *V* in Fig. 4B). T3 increased the amounts of H3K4me3 and RNAPIIS5P in the 5′ regulatory regions of both genes (panels *I* and *V* in Figs. 4A and 4B). The amounts of H3K4me3 and RNAPIIS5P in the coding regions were significantly lower than that in the 5′ regulatory regions (panels *II*–*IV* versus panel *I* and panels *VI*–*VIII* versus panel *V* in Figs. 4A and 4B).

Effects of IOX and TBBPA on Trimethylation of Histone H3K36 and Phosphorylation of RNAPIIS2 in Direct TH-Response Genes

Although T3 treatment significantly increased the amounts of H3K36me3 in the coding regions of both genes (panels *III* and *IV* in Fig. 5A, and panels *II*–*IV* in Fig. 5B), IOX and TBBPA suppressed these increases at some sites in the coding regions (panel *III* in Fig. 5A for IOX; panel *III* in Fig. 5A and panels *III* and *IV* in Fig. 5B for TBBPA). In the *thibz* gene, IOX exhibited no effects on the T3-induced increases in the amounts of H3K36me3 (panels *II*–*IV* in Fig. 5B). The higher amounts of H3K36me3 were localized on the coding regions compared with the 5′ regulatory regions (panels *II*–*IV* versus panel *I* in Figs. 5A and 5B).

T3 treatment increased the amounts of RNAPIIS2P in the 5′ regulatory and some of the coding regions of both genes (panels *V* and *VII* in Fig. 5A; panels *V* and *VI* in Fig. 5B), and IOX and TBBPA suppressed the T3-induced increase at a site in the coding region of the *thrb* gene (panel *VII* in Fig. 5A), but not in the *thibz* gene (panels *V* and *VI* in Fig. 5B).

In the TH-unresponsive gene, *rpl8*, there were no effects of TH and the chemicals on the amounts of H3K4me3, H3K36me3, RNAPIIS2P and RNAPIIS5P (data not shown).

DISCUSSION

In the present study, we demonstrated that IOX and TBBPA suppressed some of the T3-induced histone and RNAPII post-translational modifications in direct TH-response genes in *X. laevis* cells. To verify the actions of the chemicals on cellular TH-signaling pathway, we first confirmed the previous finding that the phenolic compounds, IOX and TBBPA, exerted TH-disrupting activity (Sugiyama *et al*., 2005b; Kudo *et al*., 2006) by suppressing T3-induced increase in transcript abundance of the direct TH-response *thrb* and *thibz* genes in a dose-dependent manner. ChIP assays showed that T3 increased the amounts of H4Ac, H3K4me3 and RNAPIIS5P in the 5′ regulatory regions of the *thrb* and *thibz* genes, and the amounts of H3K36me3 and RNAPIIS2P in the coding regions. Some of these T3 effects were partially in accordance with previous reports in different amphibian species (Bilesimo *et al*., 2011; Mochizuki *et al*., 2012). These changes indicate that TH activates both the early and progressive stages of RNAPII transcriptional elongation. Therefore, IOX and TBBPA may suppress the TH-induced activation of RNAPII transcriptional elongation in direct TH-response genes.

Histone Acetylation, RNAPII recruitment and Transcriptional Activation

In this study, we found chemical-insensitive and -sensitive stages in the TH-signaling pathway after T3 binds to TH receptors on TREs in direct TH-response genes (Fig. 6). Chemical-insensitive stages include H4Ac and the recruitment of RNAPII, both of which occur at 5′ regulatory regions near promoter, and may facilitate transcriptional initiation to early transcriptional elongation.

In general, TH-induced transcriptional activation is positively correlated with increased amounts of H4Ac, as shown in this and previous studies (Sachs and Shi, 2000; Havis *et al*., 2003; Bilesimo *et al*., 2011; Mochizuki *et al*., 2012). Increases in histone acetylation are well known as an epigenetic hallmark for gene transcriptional activation, and facilitate the recruitment of RNAPII to form the transcriptional initiation complexes (Fondell, 2013; Grimaldi *et al*., 2003). However, IOX and TBBPA did not significantly affect

the amounts of H4Ac and the recruitment of RNAPII that were induced by T3, despite both chemicals suppressing the T3-induced increases in transcript abundance of the *thrb* and *thibz* genes by 32–55%. Histone H4 acetylation and the RNAPII recruitment to regulatory regions may not be necessarily linked with gene transcript levels. A similar unparallel relationship in amounts between acetylated histones and some TH-response gene transcripts was reported in a study investigating the effects of an specific histone deacetylase inhibitor, trichostatin A (Sachs and Shi, 2000; Sachs *et al*., 2001).

Finding that TBBPA inhibited competitively [^{125}I]T3 binding to TH receptor with 10^3 times less potent than T3 (Kudo *et al*., 2006) suggests direct interaction of TBBPA with TH receptor. A recent study revealed that TBBPA (10^{-6}–10^{-5} M) interfered with the function of human TH receptor α in vitro (Lévy-Bimbot *et al*., 2012). In the presence of T3, TBBPA suppressed the recruitment of a coactivator peptide to the liganded TH receptor α. If this is the case, it is unclear why the amounts of T3-induced H4Ac in the 5′ regulatory regions of the *thrb* and *thibz* genes were not affected by TBBPA. Further studies will be needed to investigate the acetylation levels of histones other than histone H4, the other histone modifications that occur at early stages of transcription (Grimaldi *et al*., 2003), or modifications of non-histone proteins including TH receptor itself (Wang *et al*., 2011).

H3K4me3, H3K36me3 and Transcriptional Elongation of RNAPII

Chemical-sensitive stages include RNAPIIS5P and H3K4me3 at 5′ regulatory regions, hallmarks of early stage RNAPII transcriptional elongation, and RNAPIIS2P and H3K36me3 at coding regions, hallmarks of progressive stage RNAPII transcriptional elongation (Brookes and Pombo, 2009), in transactivation by T3 of at least the *thrb* gene (Fig. 6). RNAPIIS5P recruits histone H3K4 methyltransferase such as SUV39H1 whereas RNAPIIS2P recruits histone H3K36 methyltransferase such as NSD1 (Kato *et al*., 2011). These chemicals are likely to target some stage(s) in the TH-signaling pathway after TH binding to TH receptor but before increases in the amounts of RNAPIIS5P or

RNAPIIS2P, in the *thrb* gene.

Chemical-Specificity and Target Gene-Dependency of TH-Disrupting Activity

We found chemical-specificity and target gene-dependency in the suppression by IOX and TBBPA of T3-induced post-translational modifications on histone H3 and RNAPII. We had first assumed that IOX and TBBPA exerted TH-disrupting activity by different mechanisms, because frog TH receptors showed significant binding affinity for TBBPA, but not for IOX, on an in vitro [^{125}I]T3 competitive binding assay (Yamauchi *et al*., 2002; Ishihara *et al*., 2003; Shimada and Yamauchi, 2004; Kudo *et al*., 2006). Another reason is that TBBPA expressed not only a weak TH agonist activity in the absence of T3 but also a weak antagonist activity in the presence of T_3 (Kitamura *et al*., 2002; Kudo *et al*, 2006) whereas IOX suppressed T3-induced transactivation alone with no effects in the absence of T3 (data not shown) on a T3-dependent luciferase or a cell growth assay. These observations suggest that the two chemicals act on different sites in intracellular TH-signaling pathway. Nevertheless, in the *thrb* gene, IOX and TBBPA suppressed the same T3-induced post-translational modifications on the histone H3 and RNAPII (Fig. 6). In the *thibz* gene, TBBPA was effective on only the T3-induced H3K36me3, whereas IOX was ineffective on all of the post-translational modifications tested (Fig. 6). It is likely that TBBPA influences the recruitment of a methyltransferase for histone H3K36 to RNAPIIS2P or the methyltransferase activity itself in the *thibz* gene. As IOX is known to affect a broad range of biological processes including heart development (Campinho and Power, 2013) and tight junction formation and function (Leithe *et al*., 2010), it may act directly or indirectly on some transcriptional stages that could not be detected in the present study, probably after transcriptional elongation stages.

The differences in the architecture of promoters and TREs between the *thrb* and *thibz* genes may affect the interference with TH-signaling by IOX and TBBPA as well as the TH response. In contrast to the early TH-response *thrb* gene with a TATA-less promoter consisting of a unique initiator and an upstream

promoter sequence (Wong *et al*., 1998), the *thibz* gene has a TATA-box with various potential sites for transcription factors (Furlow and Brown, 1999). In addition, the *thibz* gene has a promoter with biphasic kinetics that are intermediate between early and late *Xenopus* TH-response genes (Furlow and Brown, 1999), suggesting the interaction of transcription factors other than the TH receptors. The *thrb* promoter has DR+2- (a direct repeat spaced by 2 nucleotides), DR+3-, and DR+4-type TREs in the 5′ upstream sequence and DR+4-type TRE in the 5′ untranslated region (Urnov and Wolffe, 2001) whereas the *thibz* promoter has two sets of DR+4-type TREs (Furlow and Brown, 1999), to which TRs bind with four times lower affinity than to the proximal TRβ DR+4-type TRE (Buchholz *et al*., 2005). Very recently, Grimaldi *et al*. (2013) proposed two categories of TREs from detailed ChIP studies in the *thrb* and *thibz* genes in *X. tropicalis* tail fin: TREs that bind unliganded and liganded TH receptors (*thrb* type) and TREs that only bind liganded TR (*thibz* type). These differences may form gene-specific TR-mediator complexes with different kinetics resulting in gene-dependent TH-disrupting activity of IOX and TBBPA.

In conclusion, IOX and TBBPA altered T3-induced histone H3 and RNAPII post-translational modifications that are closely linked to transcriptional elongation stages of RNAPII, but showed no effects on T3-induced histone H4 acetylation and RNAPII recruitment to 5′ regulatory regions. As far as we know, the present study is the first report showing that the chemicals interfere with T3-induced activation of transcriptional elongation stages on direct T3-response genes. Furthermore, we detected chemical-specific and target gene-dependent effects, which may reflect the differences in action sites of the chemicals and in the architectures of TH receptor-mediator complexes in the promoters and enhancers.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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FIGURE LEGENDS

FIG. 1. Diagrams of primer sets used for qPCR. The positions of primers used here are shown with structures of the *thrb* (*A*) (Shi *et al*., 1992) and *thibz* (*B*) genes (Furlow and Brown, 1999). Pairs of arrow heads indicate the approximate locations of the primer sets used for RT-qPCR (primer sets A and B) and qPCR in ChIP assay (primer sets A1 to A4 and B1 to B4). White boxes, exonic regions; solid lines, intronic and upstream untranscribed regions; broken line, unclear intron sizes; black boxes, TREs in 5′ regulatory regions; +1, transcription start site.

FIG. 2. Effect of IOX and TBBPA on transcript abundances of direct TH-response genes in XL58-TRE-Luc cells. (*A*) Thyroid hormone-dependent luciferase assay was conducted with or without 2 nM T3, in the presence or absence of indicated concentrations of IOX (*I*) or TBBPA (*II*). The vertical axes represent the luciferase activity as a magnitude of the induction (fold). (*B*) Amounts of T3-induced transcripts of the *thrb* (*I*) and *thibz* (*II*) genes were estimated by RT-qPCR as a magnitude of the induction (fold) after normalization with the *rpl8* gene expression. Cells were treated with or without (vehicle control) 2 nM T3, in the presence or absence of 1 μM chemicals. Each value is the mean ± SEM ($n = 3$). Distinct letters denote significantly different means, and were determined by a one-way analysis of variance and Fisher's least significant difference test for multiple comparisons ($p < 0.05$).

FIG. 3. Effect of IOX and TBBPA on histone H4 acetylation and recruitment of RNAPII in the *thrb* (*A*) and *thibz* (*B*) genes. Cells were treated with or without 2 nM T3, in the presence or absence of 1 μM IOX or TBBPA. Signals of ChIP were detected by qPCR following immunoprecipitation with antibodies against H4Ac (*I–IV*) and RNAPII (*V–VIII*). Primers used in qPCR are primer sets A1 and B1 (*I* and *V*), A2 and B2 (*II* and *VI*), A3 and B3 (*III* and *VII*), and A4 and B4 (*IV* and *VIII*) (see Supplementary table S1 and Fig. 1), in Figs. 3A and 3B, respectively. Each value is the mean ± SEM ($n = 3$). Distinct letters denote significantly

different means, and were determined by a one-way analysis of variance and Fisher's least significant difference test for multiple comparisons ($p < 0.05$).

FIG. 4. Effect of IOX and TBBPA on trimethylation of histone H3K4 and phosphorylation of RNAPIIS5 in the *thrb* (*A*) and *thibz* (*B*) genes. Cells were treated with or without 2 nM T3, in the presence or absence of 1 μM IOX or TBBPA. Signals of ChIP were detected by qPCR following immunoprecipitation with antibodies against H3K4me3 (*I–IV*) and RNAPIIS5P (*V–VIII*). Primers used in qPCR are primer sets A1 and B1 (*I* and *V*), A2 and B2 (*II* and *VI*), A3 and B3 (*III* and *VII*), and A4 and B4 (*IV* and *VIII*) (see Supplementary table S1 and Fig. 1), in Figs. 4A and 4B, respectively. Each value is the mean ± SEM (n = 3). Distinct letters denote significantly different means, and were determined by a one-way analysis of variance and Fisher's least significant difference test for multiple comparisons ($p < 0.05$).

FIG. 5. Effect of IOX and TBBPA on trimethylation of histone H3K36 and phosphorylation of RNAPIIS2 on the *thrb* (*A*) and *thibz* (*B*) genes. Cells were treated with or without 2 nM T3, in the presence or absence of 1 μM IOX or TBBPA. Signals of ChIP were detected by qPCR following immunoprecipitation with antibodies against H3K36me3 (*I–IV*) and RNAPIIS2P (*V–VIII*). Primers used in qPCR are primer sets A1 and B1 (*I* and *V*), A2 and B2 (*II* and *VI*), A3 and B3 (*III* and *VII*), and A4 and B4 (*IV* and *VIII*) (see Supplementary table S1 and Fig. 1), in Figs. 5A and 5B, respectively. Each value is the mean ± SEM (n = 3). Distinct letters denote significantly different means, and were determined by a one-way analysis of variance and Fisher's least significant difference test for multiple comparisons ($p < 0.05$).

FIG. 6. Possible target sites of the TH-disrupting chemicals IOX and TBBPA in TH-response gene activation. Post-translational modifications of histone and RNAPII occur during transcription including initiation, early elongation, progressive elongation and termination. Thyroid hormone stimulates

co-regulator switch from corepressor complexes with histone deacetylase activity to coactivator complexes histone acetylase activity, which induces H4Ac and RNAPII recruitment in 5′ regulatory regions of direct T3-response genes. TH also enhances the amounts of RNAPIIS5P with H3K4me3 in 5′ regulatory regions and the amounts of RNAPIIS2P with H3K36me3 in coding regions. Anti-TH actions of the chemicals in the *thrb* and *thibz* genes represent as black downward arrows. *, TBBPA was effective, but IOX not; +, TBBPA and IOX were effective in the *thrb* gene, but not in the *thibz* gene; #, TBBPA was effective in the *thrb* and *thibz* genes, but IOX was effective in the *thrb* gene alone. GTFs, general transcription factors; TR, TH receptor; TRE, TH-response element.

Supplementary table S1. The list of primers used and efficiencies in quantitative polymerase chain reaction

Experiment/Gene/Primer set	Sequence, 5'→3'	Position (nt)	Size amplified (nt)	Slope	R-squared	Efficiency (%)	Accession no./reference
RT-qPCR							
thyroid hormone receptor beta (*thrb*)							
Primer A	Fwd: CAAGCACCAAGAACGAAAACC	15 ~ 35 in exon f	93	-3.2651	0.9929	102.4	AH002493,
	Rev: ATTGGAAGGTCTGCTCATTCTTCTA	40 ~ 16 in exon g					Yaoita et al. (1990)
thyroid hormone induced bZip protein (*thibz*)							
Primer B	Fwd: GTTTCCGTTTTGGCCTGGTA	413 ~ 432	75	-3.2039	0.9926	105.2	NM_001085805
	Rev: CATGAAGGCCACACTGTGTTG	487 ~ 467					
ribosomal protein L8 (*rpl8*)							
Primer C	Fwd: TTGCAGCTGAGGGAATCCA	288 ~ 306	57	-3.474	0.996	94	NM_001086996
	Rev: GCTGAGCTTTCTTGCCACAGT	344 ~ 323					
glyceraldehyde-3-phosphate dehydrogenase (*gapdh*)							
Primer D	Fwd: CTCATGACAACAGTCCATGCTTTC	558 ~ 581	82	-3.4244	0.9938	95.9	NM_001087098
	Rev: CTCTGCCATCTCTCCACAGCTT	639 ~ 618					
ChIP assay							
thyroid hormone receptor beta (thrb)							
Primer A1	Fwd: CCCCTATCCTTGTTCGTCCTC	239 ~ 259	53	-3.3261	0.9881	102.6	Z38008, Wong *et al*. (1998)
	Rev: GCGCTGGGCTGTCCT	291 ~ 277					
Primer A2	Fwd: AAGATGAGCTATGTGTGGTAT	167 ~ 187	66	-3.3892	0.9932	97.3	M35362
	Rev: CGCAGGTGATACATCTATAG	232 ~ 213					
Primer A3	Fwd: AACCTCCACCCAAGCTATTCC	271 ~ 291	75	-3.4447	0.9541	95.1	M35362
	Rev: TTGGCACTGGTTCCTTGTTAC	345 ~ 325					
Primer A4	Fwd: GGTTTTGGATGACAGCAAAC	393 ~ 412	82	-3.3135	0.9965	100.4	M35362
	Rev: CTGTATCTCGTCTTTCCGTC	474 ~ 455					
thyroid hormone induced bZip protein (*thibz*)							
Primer B1	Fwd: GGACGCACTAGGGTTAAGTAAGG	-109 ~ -87	63	-3.1784	0.9906	106.4	AF192491, Furlow and Brown (1999)
	Rev: TCTCCCAACCCTACAGAGTTCAA	-47 ~ -69					
Primer B2	Fwd: CTTGGCTGTCGGAACACTTCT	169 ~ 189	64	-3.1971	0.9455	105.5	NM_001085805
	Rev: GTCGCCGGGAATTGCA	232 ~ 217					
Primer B3	Fwd: CGGCGACGGTGGGAAT	226 ~ 241	62	-3.3192	0.9965	100.1	NM_001085805
	Rev: CGCCTCTTGTCCCAATAGCT	287 ~ 268					
Primer B4	Fwd: GGCTGCAAAACGCTCAAGAG	300 ~ 319	58	-3.2796	0.9876	101.8	NM_001085805
	Rev: ACGGCCTTCAAGGACAAGATC	357 ~ 337					

Supplementary table S2. Detailed qPCR information

A. RT- qPCR information

Experimenal design	
Definition of experimental and control groups	Control group : Cells cultured in medium without 2 nM T3. Experimental groups : Cells cultured in medium with 2 nM T3 and in the absence or presence of 1 μM test chemicals
Number within each group	n=3 (see Fig. 2B)
Sample	
Description	*Xenopus laevis* T3-responsive XL58-TRE-Luc cells (Sugiyama et al., 2005a)
Volume/mass of sample processed	2 x10^6 cells/90 mm dish
Nucleic acid extraction	
Procedure and/or instrumentation	RNA extraction : the AGPC method (Chomczynski and sacchi, 1987)
Details of DNase or RNase treatment	After adding 1.82 U/μl DNase I, incubation for 15 min at 25 °C Used DNase I is component of High Pure RNA Tissue Kit (Roche, Tokyo, Japan)
Contamination assessment (DNA or RNA)	The Cqs of DNase-treated samples were slightly higher than those of untreated samples
Nucleic acid quantification	Concentrations, 200-500 μg/ml; volume, 100 μl
Instrument and method	Instrument, BioSpec-nano (SHIMADZU, Kyoto, Japan); Method, Instruction manual
Purity (A_{260}/A_{280})	A_{260}/A_{280} : 1.90-2.10
RNA integrity: method/instrument	28S rRNA and 18S rRNA bands were electrophoretically detected
Method :	Electrophoresis in a 1% agarose gel containing 2 M formaldehyde
Inhibition testing (C_q dilutions, spike, or other) E	
Reverse transcription	
Complete reaction conditions →	Final concentration of reaction reagents : 20 ng/μl RNA sample, 1 x Taqman TR buffer, 5.5 mM $MgCl_2$, 500 μM each dNTPs mixture, 2.5 μM Random hexamers, 0.4 U/μL RNase inhibitor, 1.25 U/μL MultiScribe reverse transcriptase
Amount of RNA and reaction volume	Amount of RNA, 200 ng; reaction volume, 10 μl
Priming oligonucleotide (if using GSP) and concentration	Random hexamers; final concentration, 2.5 μM
Reverse transcriptase and concentration	MultiScribe reverse transcriptase; final concentration, 1.25 U/μl.
Temperature and time	25°C for 10 min, 48°C for 30 min, and then 95°C for 5 min
Manufacturer of reagents and catalogue numbers	Taqman RT reagent kit Applied Biosystems (Foster City, CA, USA), Cat. No.,N8080234
C_qs with and without reverse transcription	Results of DNase treated samples. *thrb*: RT(+) 31.19, RT(-): 36.64, *thibz*: RT(+) 27.43, RT(-): 34.08
Storage conditions of cDNA	-20°C
qPCR target information	
Gene symbol	See the text and Suppl. table S1.
Sequence accession number	See Suppl. table S1.
Location of amplicon	See Suppl. table S1
Amplicon length	See Suppl. table S1
In silico specificity screen (BLAST, and so on)	Confirmation of primer specificity using BLAST
What splice variants are targeted?	*thrb* (primer A), *thrb*A1-6 (Yaoita *et al.* 1990); *thibz* (primer B), unknown
PCR alogonucleotides	
Primer sequences	See Suppl. table S1
qPCR protocol	
Complete reaction conditions	Final volume of reaction reagents: 12.5 μl 2 x Power SYBR Green Master Mix 2 μl 2.5 μM each Primer mix 8.5 μl Diethylpyrocarbonate-treated water
Reaction volume and amount of cDNA/DNA	Reaction volume, 25 μl; cDNA mixture after reverse transcription, 2 μl
Primer, (probe), Mg^{2+}, and dNTP concentrations	Primers, each 400 nM (final conc.) Power SYBR Green Master Mix includes Mg^{2+} and dNTP, whose concentrations are unclear
Polymerase identity and concentration	Power SYBR Green Master Mix includes AmpliTaq Gold DNA Polymerase, whose concentration is unclear.
Buffer/kit identity and manufacturer	Power SYBR Green Master Mix, Applied Biosystems (Foster City, CA, USA), Cat. No. 4367659
Exact chemical composition of the buffer	Unclear

Additives (SYBR Green I, DMSO, and so forth)	Power SYBR Green Master Mix includes SYBR Green I and Passive reference, whose concentrations are unclear.
Manufacturer of plates/tubes and catalog number	ABgene PCR Detection plates (Thermo Scientific, Yokohama, Japan). Cat. No. AB-1100
Complete thermocycling parameters	95°C for 10 min, and 40 cycles of 95°C for 15 s, 60°C for 1 min, and 50°C for 2 min
Manufacturer of qPCR instrument	ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster city, CA, USA)

qPCR validation

Specificity (gel, sequence, melt, or digest)	single band on agarose gel, sequence check to identify the amplicon, and Single peak of melting curve
For SYBR Green I, C_q of the NTC	
PCR efficiency calculated from slope	See Suppl. table S1
r^2 of calibration curve	See Suppl. table S1
Linear dynamic range	1:1/2:1/4:1/8:1/16
C_q variation at LOD	Ex) 34.03 – 35.28
Evidence for LOD	No significant difference between Cqs of diluted sample and that of NTC

Data analysis

qPCR analysis program (source, version)	ABI PRISM 7000 SDS software version 1.0 (build 81 rev3)
Method of C_q determination	Following the instruction manual, Cqs were arbitrarily determined the appropriate position.
Outlier identification and disposition	None
Results for NTCs	primer A, Cq = 38.2; primer B, Cq =36.8; primer C, Cq = 35.4; primer D, Cq = 31.4
Justification of number and choice of reference genes	Two genes *rpl8* and *gapdh* were tested. The variation in Cqs of the *rpl8* was less than that of the *gapdh*. Cq = 23.66 – 24.75 for *gapdh*; Cq = 21.66 – 21.85 for *rpl8*
Description of normalization method	The $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001)
Number and stage (reverse transcription or qPCR) of technical replicates	3 times every steps (RNA extraction, reverse transcription and qPCR)
Repeatability (intraassay variation)	In triplicates
Statistical methods for results significance	Fisher's PLSD
Software (source, version)	Microsoft Excel 2003 Data Analysis

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B. qPCR information for ChIP assay

Experimenal design

Definition of experimental and control groups	Control group: Cells cultured in medium without 2 nM T3. Experimental groups: Cells cultured in medium with 2 nM T3 and in the absence or presence of 1 μM test chemicals.
Number within each group	n=3, (see legends of Figs.3-5)

Sample

Description	*Xenopus laevis* T3-responsive XL58-TRE-Luc cells (Sugiyama et al., 2005a)
Volume/mass of sample processed	2 x10^6 cells/90 mm dish

Nucleic acid extraction

Procedure and/or instrumentation	Phenol-chloroform extraction.
Name of kit and details of any modifications	No kits were used

qPCR target information

Gene symbol	See the text and Suppl. table S1
Sequence accession number	See Suppl. table S1
Location of amplicon	See Suppl. table S1
Amplicon length	See Suppl. table S1
In silico specificity screen (BLAST, and so on)	Confirmation of primer specificity using BLAST

PCR alogonucleotides

Primer sequences	See Suppl. tableS1

qPCR protocol

Complete reaction conditions	Final volume of reaction regents : 12.5 μl 2 x Power SYBR Green Master Mix 2 μl 2.5 μM each Primer mix 8.5 μl Diethylpyrocarbonate-treated water
Reaction volume and amount of cDNA/DNA	Reaction volume, 25 μl DNA amounts corresponding to 1.0 x 10^4 cells were used for input samples DNA amounts corresponding to 4.5 x 10^4 cells were used for ChIP samples
Primer, (probe), Mg_2_, and dNTP concentrations	Each 400 nM (final conc.) Power SYBR Green Master Mix include Mg^{2+} and dNTP, whose concentrations are unclear
Polymerase identity and concentration	Power SYBR Green Master Mix include AmpliTaq Gold DNA Polymerase, whose concentration is unclear.
Buffer/kit identity and manufacturer	Power SYBR Green Master Mix, Applied Biosystems (Foster City, CA, USA), Cat. No. 4367659
Exact chemical composition of the buffer	Unclear
Additives (SYBR Green I, DMSO, and so forth)	Power SYBR Green Master Mix includes SYBR Green I and Passive reference, whose concentrations are unclear.

Manufacturer of plates/tubes and catalog number	ABgene PCR Detection plates (Thermo Scientific, Yokohama, Japan). No : AB-1100
Complete thermocycling parameters	95°C for 10min, and 40 cycles of 95°C for 15 s, 60°C for 1 min, and 50°C for 2min
Manufacturer of qPCR instrument	ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster city, CA, USA)

qPCR validation

Specificity (gel, sequence, melt, or digest)	single band on agarose gel, sequence check to identify the amplicon, and Single peak of melting curve
For SYBR Green I, C_q of the NTC	
Calibration curves with slope and y intercept	See Suppl. table S1
PCR efficiency calculated from slope	See Suppl. table S1
r^2 of calibration curve	See Suppl. table S1
Linear dynamic range	1:1/2:1/4:1/8:1/16
C_q variation at LOD	Ex) 34.03 – 35.28
Evidence for LOD	No significant difference between Cqs of diluted sample and that of NTC

Data analysis

qPCR analysis program (source, version)	ABI PRISM 7000 SDS software version 1.0 (build 81 rev3)
Method of C_q determination	Following the instruction manual, Cqs were arbitrarily determined the appropriate position.
Outlier identification and disposition	None
Results for NTCs	primers A1-A4 and B1-B4 : Cq > 40
Description of normalization method	The formula 2^{-Cq}. The Cq values of the ChIP signals were expressed as percentages of the ChIP signals for the input DNA.
Number and stage (reverse transcription or qPCR) of technical replicates	3 times ChIP assay and qPCR
Repeatability (intraassay variation)	In triplicate
Statistical methods for results significance	Fisher's PLSD
Software (source, version)	Microsoft Excel 2003 Data Analysis

Figure A

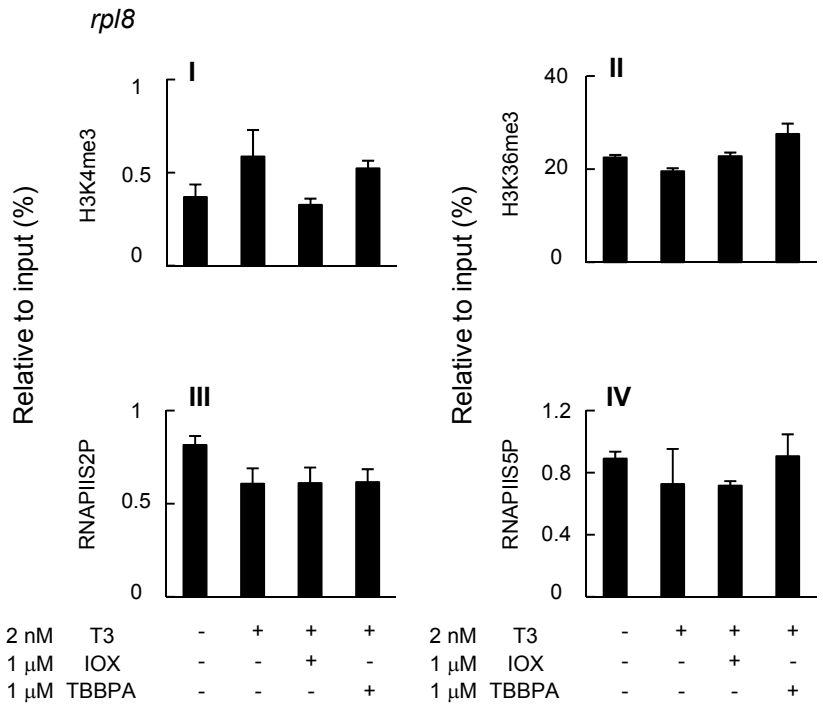


Figure A. Effects of T3, IOX and TBBPA on trimethylation of histone H3K4 and H3K36 and phosphorylation of RNAPIIS2 and RNAPIIS5 in the *rpl8* gene. XL58-TRE-Luc cells were treated with or without 2 nM T3, in the presence or absence of 1 μM IOX or TBBPA, for 24 h. Signals of ChIP were detected by qPCR following immunoprecipitation with specific antibodies against H3K4me3 (*I*), H3K36me3 (*II*), RNAPIIS2P (*III*), and RNAPIIS5P (*IV*). Primer sets used in qPCR are 5'-GAGAAAAGGTGCAGGCTCTGTT-3' (Fwd: 81-102; ac. no. NM_001086996) and 5'-CGATAGCCCGAAGCTTAGCA-3' (Rev:155-136), and 5'-GGTCGTATTGACAAACCCATCCT-3' (Fwd: 574-596) and 5'-CGTGGCCAGCAGTTTCTCTT-3' (Rev:651-632). Each value is the mean ± SEM (n = 3). There were no significant differences in the amounts of immunoprecipitated proteins among the four groups, which were determined by a one-way analysis of variance and Fisher's least significant difference test for multiple comparisons.

Figure B

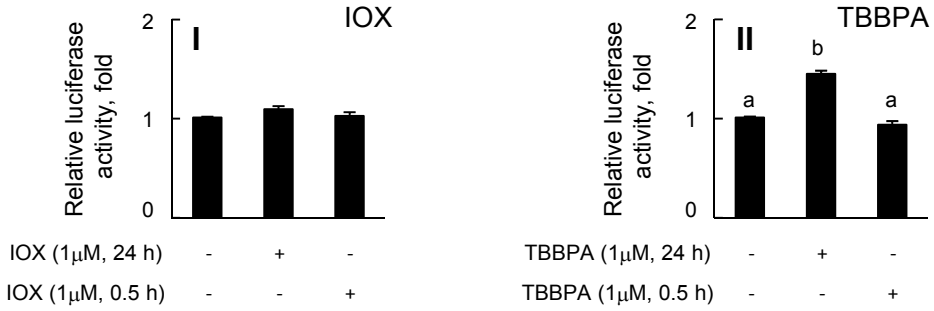


Figure B. Effect of IOX and TBBPA on luciferase activity in XL58-TRE-Luc cells. Luciferase assay was conducted without T3 in the presence or absence of indicated concentration of IOX (*I*) or TBBPA (*II*), to investigate the chemical effects on the basal level of the luciferase activity. Cells were exposed to the chemicals for 24 h or 0.5 h. The vertical axes represent the luciferase activity relative to the control (in the absence of chemicals). Each value is the mean ± SEM ($n = 3$). Distinct letters denote significantly different means, and were determined by a one-way analysis of variance and Fisher's least significant difference test for multiple comparisons ($p < 0.05$). Treatment of TBBPA for 24 h, but not for 0.5 h, increased significantly the luciferase activity, whereas treatment of IOX for both 0.5 and 24 h showed no effects.

Figure C

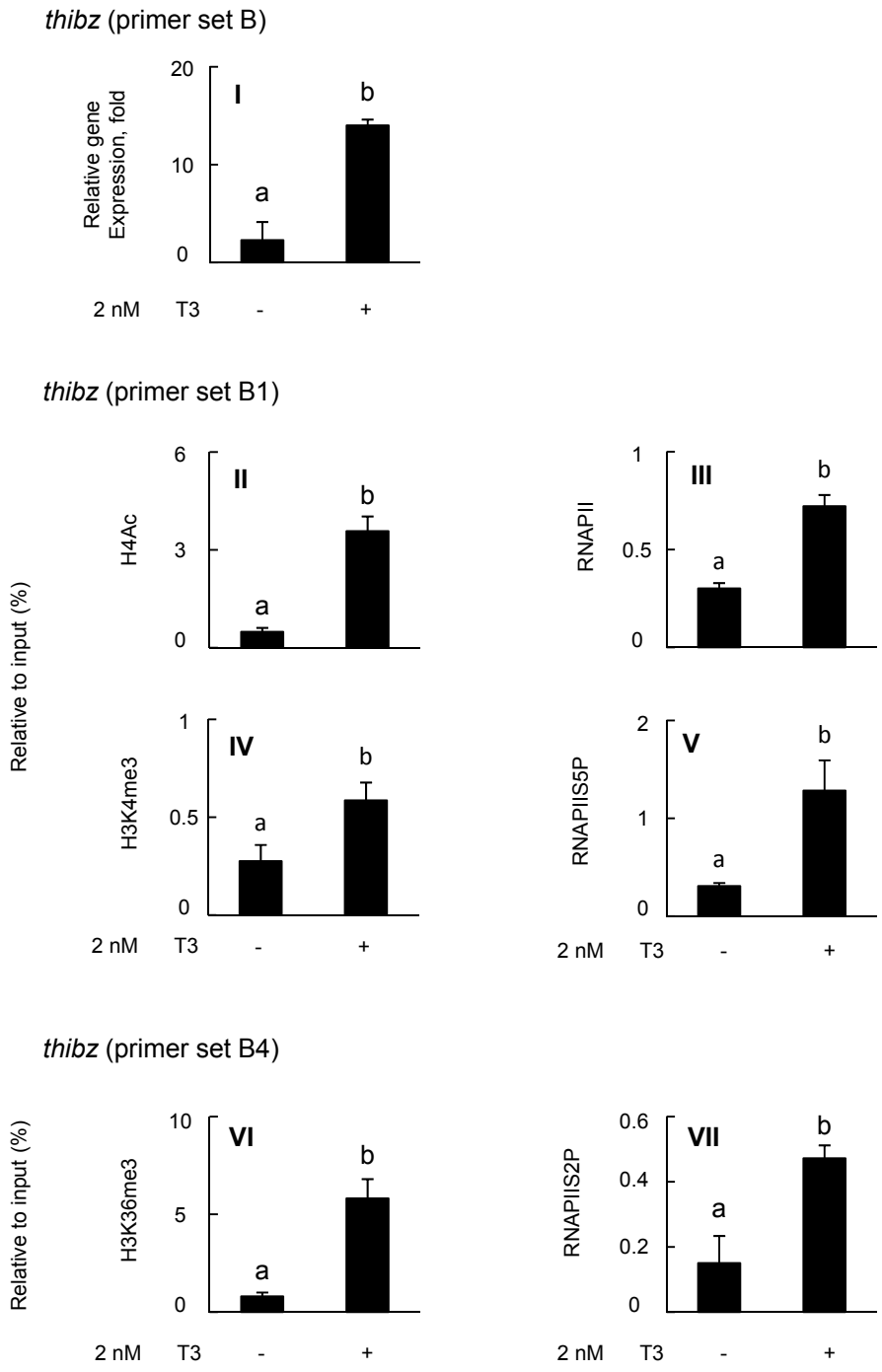


Figure C. Effect of T3 on the *thibz* transcript abundance and post-translational modifications of histones and RNAPII in XL58 cells. Amount of T3-induced transcript of the *thibz* genes (*I*)were estimated by RT-qPCR as a magnitude of the induction (fold) after normalization with the *rpl8* gene expression. Signals of ChIP were detected by qPCR following immunoprecipitation with antibodies against H4Ac (*II*), RNAPII (*III*), H3K4me3 (*IV*), RNAPIIS5P (*V*), H3K36me3 (*VI*) and RNAPIIS2P (*VII*). Primers used in qPCR are primer sets B (*I*), B1 (*II* to *V*), and B4 (*VI* and*VII*) (see Supplementary table S1 and Fig. 1), in Figs. 3A and 3B, respectively. Cells were treated with or without (vehicle control) 2 nM T3. Each value is the mean ± SEM ($n = 3$). Distinct letters denote significantly different means, and were determined by a one-way analysis of variance and Fisher's least significant difference test for multiple comparisons ($p < 0.05$).

Fig. 1

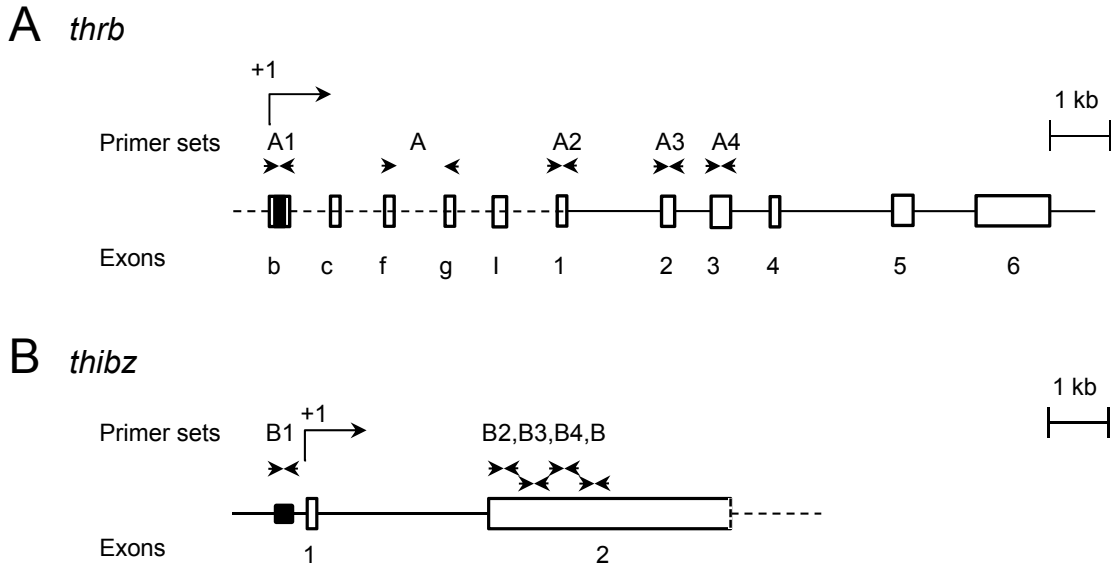

A thrb
+1
1 kb
Primer sets
A1
A
A2
A3
A4
Exons
b
c
f
g
I
1
2
3
4
5
6
B thibz
1 kb
Primer sets
B1
+1
B2,B3,B4,B
Exons
1
2

Fig. 2

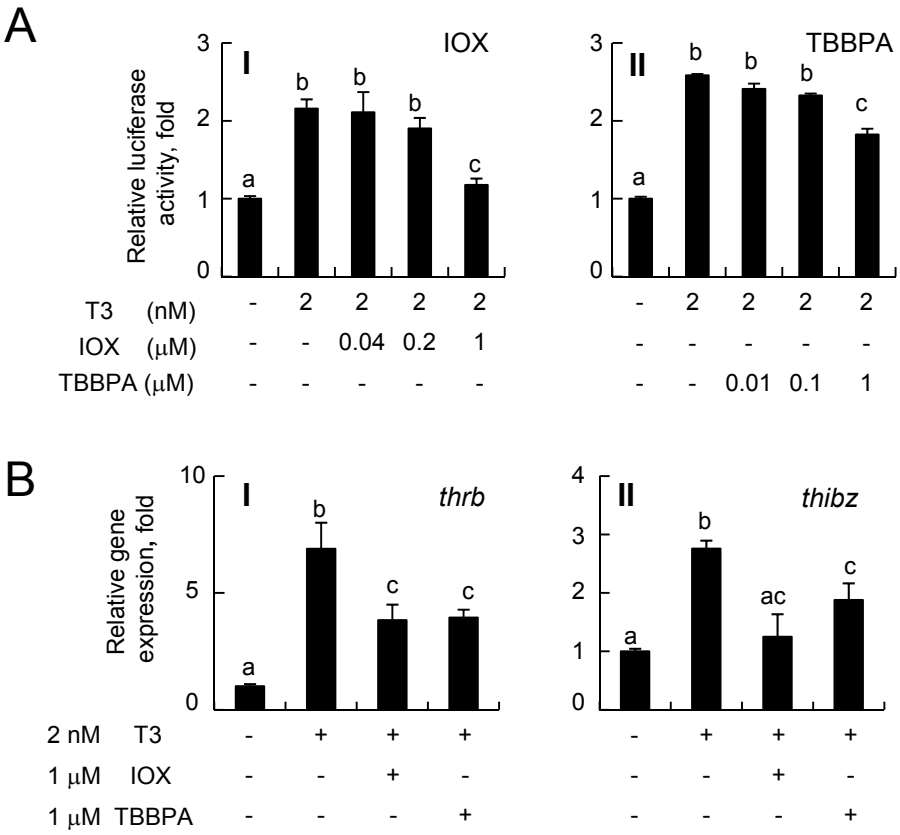

A
I
IOX
II
TBBPA
Relative luciferase activity, fold
T3 (nM)
IOX (μM)
TBBPA (μM)
B
I
thrb
II
thibz
Relative gene expression, fold
2 nM T3
1 μM IOX
1 μM TBBPA

Fig. 3

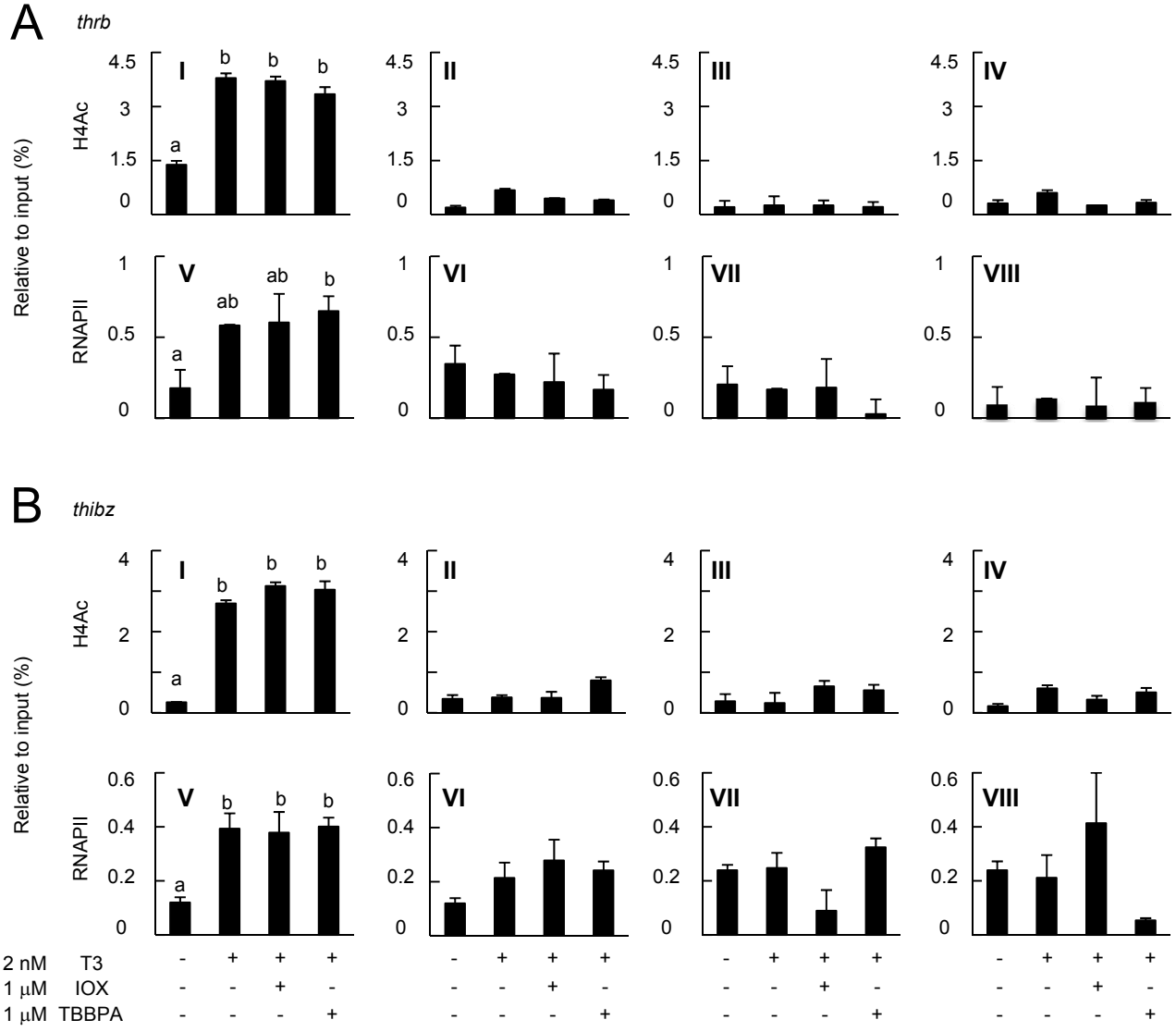

A
thrb
Relative to input (%)
H4Ac
RNAPII
I
II
III
IV
V
VI
VII
VIII
a
b
ab
B
thibz
2 nM T3
1 μM IOX
1 μM TBBPA

Fig. 4

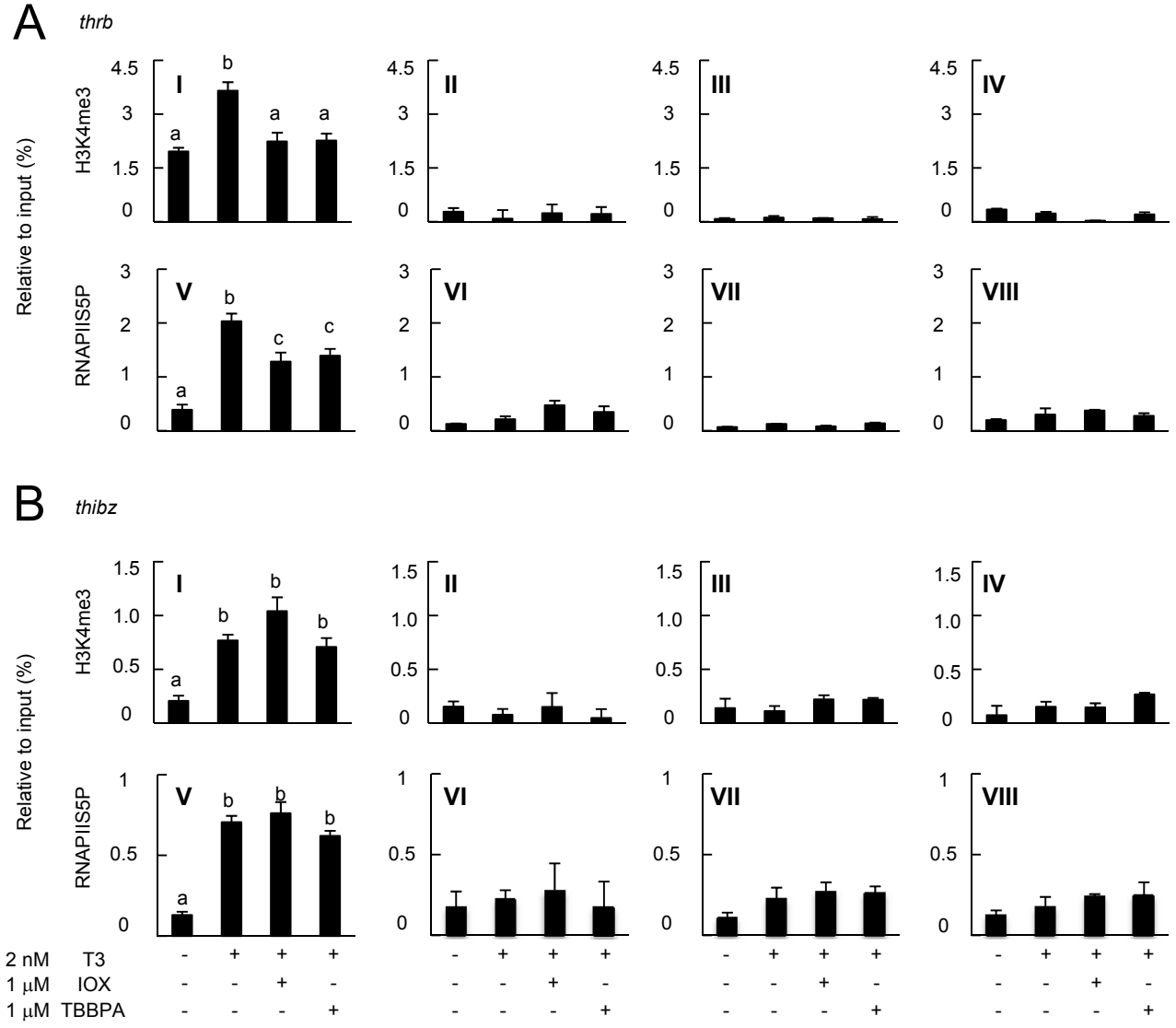

A
thrb
Relative to input (%)
H3K4me3
RNAPIIS5P
I
II
III
IV
V
VI
VII
VIII
B
thibz
2 nM T3
1 μM IOX
1 μM TBBPA

Fig. 5

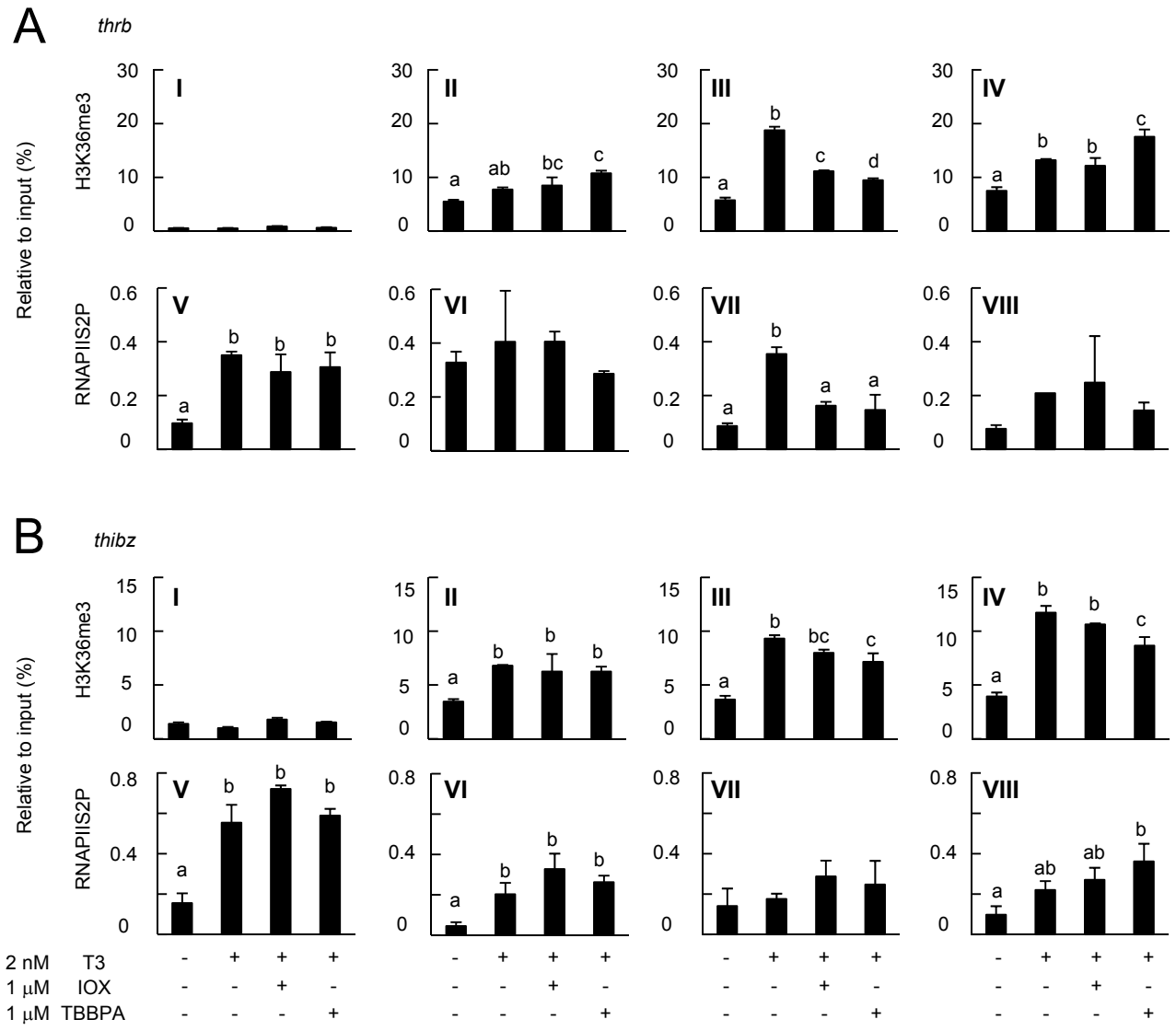
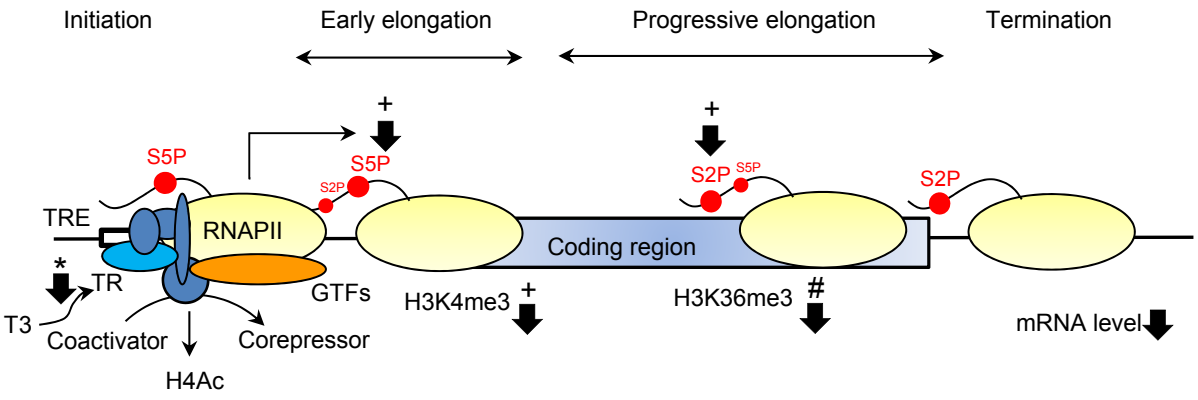

A
thrb
Relative to input (%)
H3K36me3
RNAPIIS2P
I
II
III
IV
V
VI
VII
VIII
B
thibz
2 nM T3
1 μM IOX
1 μM TBBPA

Fig. 6


Initiation
Early elongation
Progressive elongation
Termination
+
+
S5P
S5P
S2P
S2P
S5P
S2P
S2P
TRE
RNAPII
Coding region
*
TR
GTFs
H3K4me3
+
H3K36me3
#
mRNA level
T3
Coactivator
Corepressor
H4Ac