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Exposure to 3,3',5-triiodothyronine affects histone and RNA polymerase II modifications, but not DNA methylation status, in the regulatory region of the *Xenopus laevis* thyroid hormone receptor βA gene

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Abstract

Thyroid hormones (THs) play a critical role in amphibian metamorphosis, during which the TH receptor (TR) gene, thrb, is upregulated in a tissue-specific manner. The *Xenopus laevis* thrb gene has 3 TH response elements (TREs) in the 5' flanking regulatory region and 1 TRE in the exon b region, around which CpG sites are highly distributed. To clarify whether exposure to 3,3',5-triiodothyronine (T3) affects histone and RNA polymerase II (RNAPII) modifications and the level of DNA methylation in the 5' regulatory region, we conducted reverse transcription-quantitative polymerase chain reaction, bisulfite sequencing and chromatin immunoprecipitation assay using *X. laevis* cultured cells and premetamorphic tadpoles treated with or without 2 nM T3. Exposure to T3 increased the amount of the thrb transcript, in parallel with enhanced histone H4 acetylation and RNAPII recruitment, and probably phosphorylation of RNAPII at serine 5, in the 5' regulatory and exon b regions. However, the 5' regulatory region remained hypermethylated even with exposure to T3, and there was no significant difference in the methylation status between T3-untreated and -treated DNA from cultured cells or tadpole tissues. Our results demonstrate that exposure to T3 induced euchromatin-associated epigenetic marks by enhancing histone acetylation and RNAPII recruitment, but not the level of DNA methylation, in the 5' regulatory region of the *X. laevis* thrb gene.

Keywords: thyroid hormone, thyroid hormone receptor gene, histone modification, DNA methylation, *Xenopus laevis.*

1. Introduction

Amphibian metamorphosis is a dynamic post-embryonic development phase that is controlled by thyroid hormones (THs). The level of THs in blood gradually increases during prometamorphic stages and reach a peak during metamorphic climax stages [1]. Most of the TH actions are mediated by nuclear TH receptors (TRs), whose expression levels are also enhanced by the autoinduction of TR genes during metamorphosis [1]. Analyses of the promoter region of the *Xenopus laevis* TRβA gene (thrb) revealed the presence of several TH response elements (TREs) [2,3]. The most frequently characterized TRE is a direct repeat of the half site, (A/G)GGT(C/G/A)A [4], with a 4 base pairs (bp) spacer (DR4) between half sites. A dual function model has been proposed for TRs in which liganded TRs, either as a hetero- or homodimer, activate the transcription of target genes through association with coactivator complexes that have histone acetyltransferase activity whereas unliganded TRs suppress the transcription through association with corepressor complexes that have histone deacetylase activity [5].

Detailed analyses using chromatin immunoprecipitation (ChIP) assay indicated that histone modifications such as methylation and acetylation [6-9] and RNA polymerase II (RNAPII) phosphorylation [10] have important roles in TR-mediated gene activation or suppression during metamorphosis in a gene-specific or tissue-specific manner. In contrast, the role of methylation at CpG dinucleotides, another type of epigenetic modification, in TH-response genes in amphibians during metamorphosis is unclear, even though much information has been accumulated demonstrating that hypermethylation of the promoter of the TRβ1 gene correlates with transcriptional silencing in human cancers [11-13]. In general, methylation of CpG sites is primarily associated with transcriptional repression [14]. In *X. laevis,* genomic DNA is hypermethylated in oocytes and early developmental stages, and gradually demethylated at neural tube stages [15]; however, information about the level of DNA methylation at post-embryonic stages is lacking.

This study was conducted to examine whether TH affects the level of DNA methylation and other epigenetic marks on histones and RNAPII in the promoter region of the thrb gene in a *X. laevis* cell line or tadpole tissues. The cultured cells and tadpoles were treated with or without TH for 1 and 2 days, respectively. We investigated the thrb gene expression by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), post-translational modifications on histone H3 at lysine 4 (H3K4; methylation) and H4 (acetylation) and RNAPII at serine 5 (RNAPIIS5; phosphorylation) by ChIP assay, and the level of DNA methylation by bisulfate-sequencing.

2. Materials and Methods

2.1. Reagents and antibodies.

3, 3',5-Triiodothyronine (>98% purity) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against RNAPII (Cat. No. MMS-126R) and normal rabbit immunoglobulin G (15006) were purchased from Covance (Berkeley, CA, USA) and Sigma-Aldrich, respectively. Antibodies against trimethylated histone H3 lysine 4 (trimethylated H3K4; ab1012) and phosphorylated RNAPII serine 5 (phosphorylated RNAPIIS5; ab5131) were obtained from Abcam (Tokyo, Japan). Antibody against acetylated histone H4 that recognizes acetylated lysines 5, 8, 12 and 16 (06-598) was purchased from Merck Millipore (Darmstadt, Germany).

2.2. Cell line and luciferase reporter assay

Recombinant XL58-TRE-Luc cells, containing the firefly luciferase gene under the control of the SV40 promoter and *X. laevis* TREs, were used for the luciferase assay as described previously [16]. In brief, 10^5 cells were incubated on a 24-well plate in 70% Leibovitz's L-15 medium containing 10% resin-stripped fetal bovine serum [17]. The sub-confluent cells were cultured in fetal bovine serum-free 70% Leibovitz's L-15 medium for 6 h followed by culture in serum-free 70% Leibovitz's L-15 medium with or without 2 nM T3 for 24 h. The cell lysate was assayed for firefly luciferase activity using the PicaGene Luminescence Kit (Nippon Gene, Tokyo, Japan).

2.3. Animals

African clawed frog *X. laevis* tadpoles were purchased from Watanabe Zoushoku Co. (Hyogo, Japan) and were maintained at 22−24°C in aerated and dechlorinated tap water. The tadpoles were classified according to their developmental stage as outlined by Nieuwkoop and Faber [18]. After acclimation for 2 weeks, the tadpoles were reared in 3 aquaria (5 animals/L FETAX medium/aquarium) in the presence of 2 nM T3 or the same amount of dimethyl sulfoxide (final concentration 0.003%) at 22−24°C for 2 days. The rearing medium was renewed every 12 h. Tadpoles were not fed during the experiment. At the end of the experiment, tadpoles were anesthetized with 0.02% 3-amonobenzoic acid ethyl ester (Sigma-Aldrich) and their tail and intestine were dissected, in accordance with the code of ethics from the Animal Welfare Committee of Shizuoka University.

2.4. RNA extraction and RT-qPCR.

Total RNA was extracted by the acid-guanidinium-thiocyanate-phenol-chloroform method [19]. RNA was photometrically quantified by a UV spectrometer (BioSpec-nano, Shimadzu, Kyoto, Japan). To confirm its integrity, RNA (1 μg per lane) was electrophoresed in a 1% agarose gel containing 2.1 M formaldehyde, and 28S

and 18S rRNAs were visualized by ethidium bromide staining. RNA (1 μg) was treated with reverse transcriptase (SuperScript® III Reverse Transcriptase, Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. The transcript amounts of genes were estimated in triplicate using the Power SYBR® Green Master Mix and ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with a specific primer set (each 200 nM), as described previously [20]. The PCR program included a step of denaturation at 50°C (2 min) and 95°C (5 min), and 40 cycles of 95°C (15 s) and 60°C (1 min). The amount of thrb transcript was quantified using the formula $2^{-\Delta\Delta Cq}$ [21], where the endpoint, Cq, was defined as the PCR cycle number that crossed an arbitrarily placed signal threshold. To standardize each experiment, the amount of thrb transcript was divided by the amount of ribosomal protein L8 (rpl8) or elongation factor 1α (eef1a1) transcript. The primer sequences used were as follows: *X. laevis* thrb transcript (accession number, M35356 and M35357) sense 5'-GCTGCCGCTTCTACTTCAGTTC-3' (nucleotide numbers 63−84 in exon b) and antisense 5'-GCCTGCTTAACCCCAATCAGT-3' (30−10 in exon c); *X. laevis* rpl8 transcript (NM_001086996) sense 5'-TTGCAGCTGAGGGAATCCA-3' (288−306) and antisense 5'-GCTGAGCTTTCTTGCCACAGT-3' (344−323); and *X. laevis* eef1a1 transcript (NM_001087442) sense 5'-CCATTGAAAAGTTCGAGAAGGAA-3' (159−181) and antisense 5'-TCCAAGACCCAGGCATACTTG-3' (228−208). PCR efficiency was 106% for thrb, 94% for rpl8, and 84% for eef1a1.

2.5. Isolation of genomic DNA and bisulfite sequencing

Total genomic DNA was prepared from *X. laevis* cultured cells (10^7 cells) and tissues (20−30 mg) using a kit (NucleoSpin® Tissue, Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. DNA was photometrically quantified by a UV spectrometer. The amount of DNA recovered was 10−12 μg DNA from the cultured cells and 15−20 μg from the tissues. The DNA (400 ng) was sonicated to obtain approximately 400-bp fragments and then treated with bisulfite using a kit (MethylEasy™ Xceed Rapid DNA Bisulphite Modification Kit, Takara, Shiga, Japan).

Bisulfite sequencing was performed as described elsewhere [22]. The primer set B1 used for PCR amplification of bisulfite-modified DNAs was 5'-AATAAACCCTCAACCTAAAA-3' for thrb promoter forward (-746 to -727) and 5'-AGTGAAGATTTATAAGGGTT-3' for thrb promoter reverse (-271 to -290) [23]. The PCR amplicons, which originally contained 18 CpG, were ligated into pMD20 (Takara) or pGEM vector (Promega, Madison, WI, USA), and 10−12 clones independently isolated from each transformation were sequenced.

2.6. Chromatin immunoprecipitation assay

The chromatin immunoprecipitation assay was performed as described previously [20]. In brief, chromatin

in cultured cells or tissues was cross-linked in 7.7 mL or 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) 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 or tissues were washed twice with 10 mL of FACS solution (1× phosphate buffered saline, 2% bovine serum, and 0.05% sodium azide). The pelleted sample was solubilized in 1 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulfate). The lysate was sonicated (12 × 30-s pulses; Ultra5 homogenizer, VP-55, TAITEC, Saitama, Japan) to obtain DNA fragments. The diluted chromatin sample (1 mL) was mixed with a 50% Protein G Sepharose slurry (10 μL) containing 100 μg/mL salmon sperm DNA and 1% bovine serum albumin. The supernatant was used for input (50 μL) and each ChIP assay (225 μL). Chromatin solution was incubated in a rotator at 4°C overnight with each antibody (0.3−0.6 μg depending on the antibody used) or normal immunoglobulin G. The 50% Protein G Sepharose slurry (5 μL) was added to the chromatin-antibody solution. 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 (C1 to C5) to quantify the amounts of DNA. The C_q values of the ChIP signals were expressed as percentages of the ChIP signals for the input DNA. Primer sets for the thrb promoter were: 5'-GAATCGCGCCAATGTTTATT-3' (-1450 to -1431) and 5'-TTACCACTGGCTGCATTCA-3' (-1381 to -1399) (C1), 5'-CAACATCATACAGTAGTGCAGGCA-3' (-854 to -831) and 5'-CTATAATAGGCGGGCCAAGC-3' (-754 to -773) (C2), 5'-TCTGGGTTCGTCTTTTATAGATGCT-3' (-639 to -615) and 5'-TGCCGCTGATGAAGTCAC-3' (-573 to -590) (C3), 5'-GGAACTCCGAGGTAACTTCT-3' (-45 to -26) and 5'-GACTCACTGAAATGTGTG-3' (39 to 22) (C4), and 5'-CCCCTATCCTTGTTCGTCCTC-3' (238 to 258) and 5'-GCGCTGGGCTGTCCT-3' (290 to 276) (C5). PCR efficiency was 105% for C1, 100% for C2, 110% for C3, 108% for C4, and 103% for C5.

2.7. Statistical analysis

The data are presented as mean ± standard error of the mean (SEM). Differences between groups were analyzed with Student's *t*-test for comparison between two groups. $P < 0.05$ was considered statistically significant.

3. Results and Discussion

3,3',5-Triiodothyronine-dependent induction of TH-response genes was confirmed in XL58-TRE-Luc cells

and *X. laevis* tadpole tissues. Exposure to T3 (2 nM) for 1 day enhanced the amount of the thrb transcripts 19-fold and luciferase activity 2.4-fold in XL58-TRE-Luc cells (Fig. 1). Similar increases in the amount of thrb transcript were detected in the tail (~10-fold) and intestine (39-fold) of pre-metamorphic tadpoles (at stage 53) with exposure to T3 (2 nM) for 2 days.

A survey of 1.5-kbp of the 5' flanking region and the first exon (exon b) of the *X. laevis* thrb gene detected 52 CpG sites and 4 TREs (Fig. 2A). Three of the four TREs are in the 5' flanking region: TRE(DR2) (AGGGTGCCAGGTCA, -787 to -774), TRE(DR3) (TGACACTGCCGACCT, -697 to -683), and TRE(DR4) (AGGTAAGCCCGGGTTA, -523 to -508); and the other TRE is in the first exon (exon b): proximal TRE(DR4) (AGGTCATTTCAGGACA, +266 to+281) [3]. Although the retinoid X receptor (RXR)/TR heterodimer usually interacts with TRE(DR4) [24], a previous study using foot printing with DNase I and electromobility shift assay indicated that all of the three 5' flanking TREs as well as the proximal TRE in exon b were functional, with the following rank order of binding affinity for the RXR/TR heterodimer: proximal TRE(DR4) > TRE(DR4) > TRE(DR3) ≥ TRE(DR2) [3]. About half of 52 CpG sites were distributed in the 5' flanking region from -900 to -400 bp, in accord with the location of TRE(DR2), TRE(DR3), and TRE(DR4). Interestingly, TRE(DR3) and TRE(DR4) each contained one CpG site. This finding raises the possibility that the methylation status of the CpG sites in TREs may directly affect TH-specific responses. This is because exposure to estrogen and demethylation of a CpG site found in the avian vitellogenin II promoter [25] between the estrogen-response element and progesterone-response element induced the transcription of vitellogenin II gene in a tissue specific manner (in the liver) [26].

Our results suggest that the level of DNA methylation in the 5' flanking TRE region of the *X. laevis* thrb gene is not associated with TH-dependent transcriptional activation in cultured cells or pre-metamorphic tadpoles. Bisulfite-sequencing revealed that most of the CpG sites examined were highly methylated (97%), and that the level of DNA methylation did not differ between the TH-treated and -untreated cultured cells (*a* and *b* in Fig. 2B) and tadpole tails at stage 53 (*c* and *d* in Fig. 2B). A similar result was also obtained for DNA extracted from tadpole intestines at stage 53 (data not shown). Compared with the level of DNA methylation in tadpole intestines at stage 53 (*e* in Fig. 2B), the CpG site located in the 5' flanking TRE(DR3) and the 5' flanking TRE(DR4) was demethylated (4 out of 10, and 5 out of 10 clones, respectively) in tadpole intestines at stage 66 (*f* in Fig. 2B). During metamorphosis, tadpole intestine undergo rapid and dramatic tissue remodeling from a long coiled and relatively simple tubular structure with a single fold to a short and thicker tubular structure with a trough-crest axis. At a cellular level, most of the larval epithelial cells undergo apoptosis, and a small number of undifferentiated cells differentiate into a layer of adult epithelium [27]. The plasma concentration of THs, key factors in intestine remodeling, increases during prometamorphic stages, reaches a peak at climax stages 62−65,

and then declines at stage 66 [1]. Demethylation of specific CpG sites may affect the sensitivity of the thrb gene activation to THs in adult intestine after metamorphosis by changing the interaction of RXR/TR with TREs in the 5' flanking region of the thrb gene.

Our ChIP result suggests that liganded TR complex with histone acetyltransferase activity on the 5' flanking TREs and the proximal TRE may induce acetylation of histone H4, recruitment of RNAPII, and probably phosphorylation of RNAPIIS5, to activate the initiation or early elongation of thrb transcription. The levels of acetylation of histone H4 increased in the 5' flanking TRE region, specifically 2- to 3-fold for primer set C2 (from -854 to -754 bp), and 3- to 5-fold for primer set C3 (from -639 to -573 bp), and in exon b, specifically 2- to 4-fold for primer set C5 (from +238 to +290 bp) in both XL58-TRE-Luc cells and tadpole tails at stage 53 with exposure of 2 nM T3 (Figs. 3A and 3B). The level of trimethylation of histone H3K4 did not differ significantly between the TH-treated and -untreated samples in the cultured cells or tadpole tails with exposure to 2 nM T3 (Figs. 3C and 3D), although it tended to increase in exon b in the XL58-TRE-Luc cells (P=0.08). RNAPII binding to the thrb gene was enhanced 2.6-fold in the C3 region and 3.3-fold in the C5 region in XL58-TRE-Luc cells (Fig. 3E). In tadpole tails, RNAPII binding was enhanced 2- to 9-fold in all regions tested (Fig. 3F). The levels of phosphorylated RNAPIIS5 increased 2.7-fold in the C3 region and 5.0-fold in the C5 region in XL58-TRE-Luc cells and 2.9-fold in the C4 region in tadpole tails (3G and 3H). The ChIP signals for normal rabbit immunoglobulin G were less than 0.2% of input at all regions and were not significantly changed between T3-untreated and -treated samples (Figs. 3I and 3J).

The present ChIP data on T3-dependent acetylation of histone H4 and RNAPII recruitment, but not trimethylation of histone H3K4, in the C5 region were in agreement with previous studies in *X. laevis* and *X. tropicalis* tails [6,8]. In addition, our study raised the possibility that liganded TRs bound to the 5' flanking TREs also influence acetylation of histone H4 and the RNAPII recruitment. A recent study indicated that T3 induced the removal of nucleosomes at the C5 region of the thrb gene [28]. However, it is unclear whether the nucleosomes were also removed at the C2 or C3 regions containing the 5' flanking TREs.

In conclusion, T3-induced thrb gene activation is mediated by liganded TR binding to TREs in the 5' flanking and exon b regions. The present study demonstrated that exposure to T3 may affect the epigenetic modifications on histone and RNAPII but not the level of DNA methylation in the primary TH-response thrb gene. T3 induced the euchromatin-associated epigenetic marks on histone and RNAPII in in the 5' flanking and exon b regions: increases in the amounts of histone H4 acetylation, RNAPII recruitment, and probably phosphorylation of RNAPIIS5; and that the CpGs in the 5' flanking TRE region remain hypermethylated regardless of exposure to T3.

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Figure legends

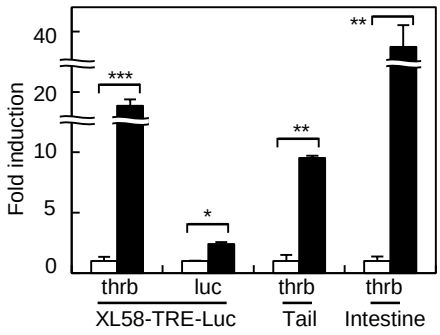
Fig. 1 Expression of thyroid hormone response genes. *Xenopus laevis* XL58-TRE-Luc cells or tadpoles at stage 53 were treated without (open bar) or with (closed bar) 2 nM 3,3',5-triiodothyronine (T3). Total RNA was extracted from the cultured cells or tadpole tissues (tails and intestines). Amounts of thrb transcript were analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The cell lysate from XL58-TRE-Luc cells was also assayed for luciferase activity (luc). The vertical axis represents the amounts of the thrb transcript or the luciferase activity as a magnitude of induction (fold induction). Each value is the mean ± standard error of the mean (SEM, n=3). *$P < 0.05$, **$P < 0.01$; ***$P < 0.001$. The experiment was repeated 3 times, with similar results.

Fig. 2. Methylation status of the thrb promoter in *Xenopus laevis* XL58-TRE-Luc cells or tadpole tissues. (*A*) Schematic representation of the 5' proximal flanking region and exon b of the thrb gene from -1558 to +377. Thin vertical lines mark the location of CpG dinucleotides. Open box, exon b; closed boxes, non-consensus thyroid hormone response elements (TREs) [3]; gray box, upstream DNA element (UPE) [23]; B1, positions of a primer set for bisulfite-sequencing; C1 to C5, positions of primer sets for chromatin immunoprecipitation (ChIP) assay. (*B*) Bisulfite DNA sequencing of the 5' flanking regulatory region from -750 to -450 bp in the thrb gene. Total genomic DNA was extracted from *X. laevis* XL58-TRE-Luc cells (*a* and *b*), tadpole tails (stage 53; *c* and *d*)

or intestines (stage 53, *e*; stage 66, *f*). XL58-TRE-Luc cells or tadpoles at stage 53 were treated without (*a* and *c*) or with (*b* and *d*) 2 nM 3,3',5-triiodothyronine (T3). Polymerase chain reaction (PCR) amplicons obtained from bisulfite-modified DNA with primer set B1 were cloned into pMD20 or pGEM vector, and at least ten individual clones from each sample were sequenced. Closed circle, methylated CpG site; open circle, unmethylated CpG site; vertical arrow, developmentally specific unmethylated CpG site detected in TRE(DR3) and TRE(DR4).

Fig. 3. Effects of exposure to 3,3',5-triiodothyronine (T3) on histone H4 acetylation, histone H3K4 trimethylation, recruitment of RNAPII and RNAPIIS5 phosphorylation in the thrb gene. *Xenopus laevis* XL58-TRE-Luc cells (*A*, *C*, *E*, *G* and *I*) or tadpoles at stage 53 (*B*, *D*, *F*, *H* and *J*) were treated without (open bar) or with (closed bar) 2 nM T3. Signals of chromatin immunoprecipitation (ChIP) were detected by quantitative polymerase chain reaction (qPCR) following immunoprecipitation with antibodies against acetylated histone H4 (*A* and *B*), trimethylated histone H3K4 (*C* and *D*), RNAPII (*E* and *F*), phosphorylated RNAPIIS5 (*G* and *H*), and normal immunoglobulin G (*I* and *J*). Primers used in qPCR are primer sets C1, C2, C3, C4 and C5. Each value is the mean ± standard error of the mean (SEM, n=3). $*P < 0.05$, $**P < 0.01$; $***P < 0.001$. P values of several non-significant increases are also shown. The experiment was repeated 3 times for cultured cells and 4 times for tadpoles, with similar results.

Fig. 1


Fold induction
40
20
10
5
0
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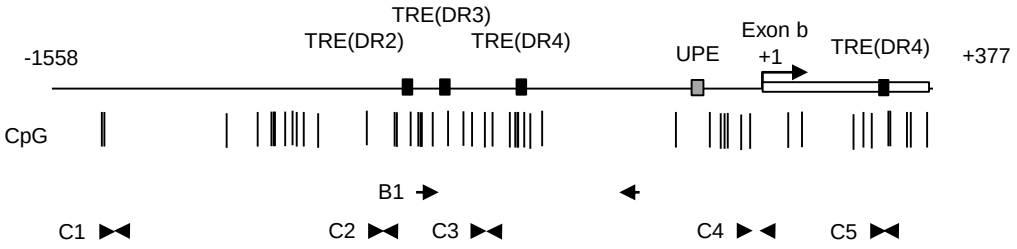
thrb
luc
thrb
thrb
XL58-TRE-Luc
Tail
Intestine

Fig. 2

A


TRE(DR3)
TRE(DR2)
TRE(DR4)
Exon b
UPE
+1
TRE(DR4)
-1558
+377
CpG
B1
C1
C2
C3
C4
C5

B

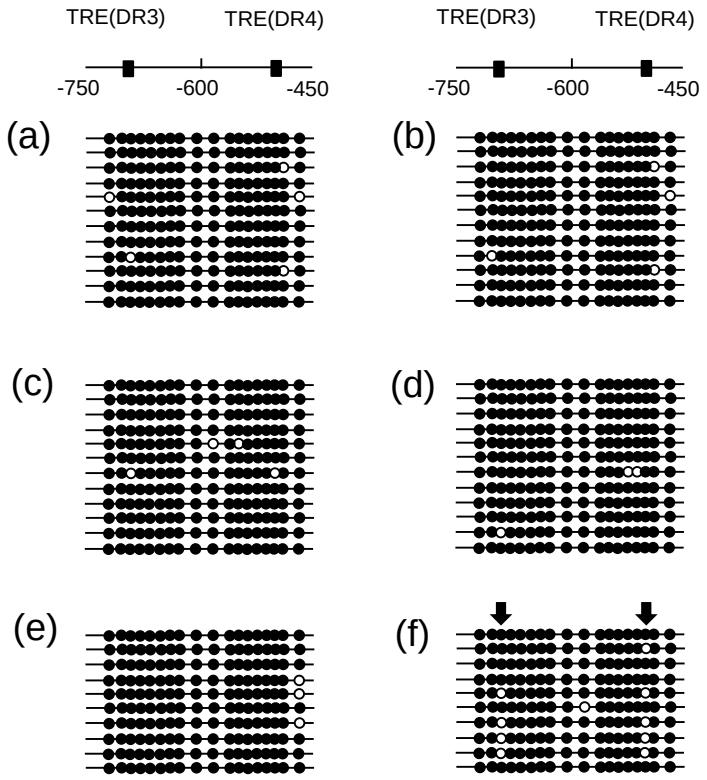

TRE(DR3)
TRE(DR4)
-750
-600
-450
TRE(DR3)
TRE(DR4)
-750
-600
-450
(a)
(b)
(c)
(d)
(e)
(f)

Fig. 3