Expression of Matrix Metalloproteinase Genes in Regressing or Remodeling Organs during the Amphibian Metamorphosis

Running Title: TREs in *Xenopus* MMP Genes

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Abstract

Several matrix metalloproteinases (MMP) are induced by thyroid hormone (TH) during the climax of amphibian metamorphosis, and play a pivotal role in the remodeling intestine and the regressing tail and gills by degrading the extracellular matrix (ECM). We compared MMP gene expression levels precisely by the quantitative real time reverse transcription-polymerase chain reaction. The expression of MMP genes increases prominently at NF stages 60, 60-61, and 62 in the intestine, gills, and tail, respectively, when the drastic morphological change starts in each organ. The gene expression analysis in the TH-treated tadpoles and cell line revealed that MMP mRNAs are up-regulated in response to TH quickly within several hours to low levels and then increase in a day to high levels. All TH-induced MMP genes have thyroid hormone response elements (TREs). The presence of high affinity TREs in MMP genes correlates with the early TH-induction. Based on these results, we propose that TH stimulates the transcription of MMP genes through TREs within several hours to low levels, and then bring about the main increase of mRNAs by TH-induced transcriptional factors including TH receptor β in cell type-specific transcriptional environment.

Key words: Amphibian metamorphosis, Matrix metalloproteinases, Thyroid hormone response element, Thyroid hormone, *Xenopus*

Introduction

The systematic and dramatic transformation is induced by thyroid hormone (TH) during the amphibian metamorphosis in order to transit from aquatic to terrestrial life style. At the climax of metamorphosis from Nieuwkoop and Faber stage (NF stage) 58 to 66 when endogenous TH levels peak, *Xenopus laevis* tadpoles undergo many morphological changes including the remodeling of intestine, skin, skeleton, pancreas, etc. and the absorption of both the tail and gills in the temporally predetermined order (Dodd & Dodd, 1976).

At NF stage 59 the lumen of the entire intestinal tract narrows progressively, and a considerable shortening of the intestinal tract takes place between NF stage 61 and 62 (Nieuwkoop & Faber, 1956, Marshall & Dixon, 1978). The larval epithelial cells undergo programmed cell death, and are replaced by the newly differentiated adult epithelial cells (Bonneville, 1963). Resorption of gills starts at NF stage 60 and is completed by NF stage 63 (Berry *et al.*, 1998a, Minnich *et al.*, 2002). In tadpole tail, the notochord begins to degenerate posteriorly at NF stage 61, and massive resorption starts around NF stage 62 when a length is reduced rapidly (Nieuwkoop & Faber, 1956).

The interactions of cells with the extracellular matrix (ECM) are critical for the normal development and function of the organism, because ECM serves as a structural support and a medium for cell-cell interactions. The matrix metalloproteinases (MMP) regulate the cell migration, the activity of biologically active molecules, and ECM microenvironment leading to cell proliferation, apoptosis, or morphogenesis by

degrading the components of ECM, and have been suggested in many physiological processes including the embryonic development, tissue resorption and remodeling, and pathological events such as tumor invasion and arthritis (Vu & Werb, 2000, Nelson *et al.*, 2000, Martel-Pelletier, 1999). The extensive degradation and reconstruction of ECM are involved in regressing or remodeling organs during the metamorphosis. In tailfins of TH-treated tadpoles, the collagenous layers and fibrils of the basement membrane begin to fray and separate, and the basement membrane is invaded by the mesenchymal cells, whose cytoplasm is filled with bundles of collagen fibrils. The basement membrane in the resorbing tail swells tenfold from a compact layer to a diffuse fragmented region filled with the large mesenchymal cells (Usuku & Gross, 1965). The basement membrane in the intestine suddenly folds vigorously into accordion-like pleats at NF stage 60, and becomes thicker, irregular, and granular till NF stage 62, while collagen fibers rapidly increase in number and density around NF stage 60 and many packed collagen fibers are observed in the entire extracellular space throughout the metamorphic climax (Ishizuya-Oka & Shimozawa, 1987).

The matrix metalloproteinases are a family of proteinases capable of degrading various components of ECM. The first reported collagenase activity is observed in skin, gut, and gills of amphibian tadpoles (Gross & Lapiere, 1962), and implied to be involved in remodeling of the connective tissue by the degradation of both collagen fibers and interfibrillar ground substance in concert with hyaluronidase activity during the metamorphosis (Eisen & Gross, 1965). The TH-induced resorption of tailfins in an organ culture is accompanied by the appearance of collagenase in medium and by

the concurrent loss of collagen from the tissues (Davis *et al.*, 1975). Furthermore, the similar result is obtained by using gills (Derby *et al.*, 1979). These experiments support the concept that collagenase plays an important role in the physiologic removal of collagen in resorbing tailfins and gills, and is controlled by TH. MMP-11 (stromelysin-3), MMP-13 (collagenase-3), and fibroblast activation protein α (FAPα) are isolated by PCR-based subtractive hybridization as ECM-degrading proteinase genes highly expressed in the regressing tail (Wang & Brown, 1993). Thereafter, MMP-18 (collagenase-4), MMP-2, MMP-9TH, and MMP-14 (the membrane-type 1 MMP) genes are reported to be up-regulated in the resorbing tail during the metamorphosis (Stolow *et al.*, 1996, Jung *et al.*, 2002, Fujimoto *et al.*, 2006, Hasebe *et al.*, 2006). These proteinase genes except for MMP-13 and FAPα are also induced in the remodeling intestine. A few of MMP genes are known to be activated in the intestine earlier than in the tail (Patterton *et al.*, 1995, Fujimoto *et al.*, 2006). It is essential and important for studying the regulation of developmental timing of MMP gene expression in morphologically changing organs to compare the expression pattern of each MMP gene quantitatively during the spontaneous and TH-induced metamorphosis.

Since exogenous TH up-regulates mRNA of thyroid hormone receptor (TR) β as much as 20-fold in a tadpole, it is hypothesized that the cascade of metamorphosis is repressed during the premetamorphosis by TRα protein binding to thyroid hormone response element (TRE) in TRβ genes, and that the increasing TH relieves this repression during development and up-regulates the expression of TRβ, which, in turn, activates the downstream genes in the cascade (Yaoita & Brown, 1990). This idea is

supported by a report that the highest expression of TRβ localizes to fibroblasts that strongly up-regulate the delayed response genes such as MMP-13 and FAPα (Berry *et al.*, 1998b). How the up-regulation of TRβ genes leads to the activation of ECM-degrading enzyme genes remains to be solved.

In the present study, we have carried out the extensive quantitative real time reverse transcription-polymerase chain reaction to examine the developmental expression profile and the TH-induced expression of each MMP gene in regressing or remodeling organs. ECM-degrading proteinase genes including both early and delayed response genes are induced simultaneously and prominently just before the transformation of a organ during the spontaneous metamorphosis, and activated in the intestine, tail and gills during the TH-induced metamorphosis and in TH-treated cultured cell line. All examined MMP and FAPα genes have TRE, to which the heterodimer of TR and 9-cis-retinoic acid receptor (RXR) binds *in vitro*. These data suggest that the expression of MMP genes is activated directly in response to TH through TRE to low levels, and then up-regulated strongly by TH-induced transcriptional factors including TRβ protein.

Materials and Methods

Animals and thyroid hormone treatment

Xenopus laevis tadpoles were staged according to Nieuwkoop and Faber (1956). The intestine, tail, and gills were dissected from tadpoles of NF stage 57 to 63 to analyze the developmental expression profiles. For the TH-induced expression analysis, tadpoles were immersed from NF stage 51-52 in 1 mM methimazole (SIGMA, St Louis, MO, USA) for more than two weeks to block the synthesis of endogenous TH, and were treated with 100 nM 3,3',5-triiodo-L-thyronine (T_3) (SIGMA) for 6, 12 and 24 h, or with 5 nM T_3 for 4 and 7 days in the presence of 1 mM methimazole. All operations were performed after animals were anesthetized with 0.02% MS-222 (SIGMA). *Xenopus tropicalis* tadpoles (Nigerian line) were the generous gifts of the National Bioresource Project (Institute for Amphibian Biology, Hiroshima University). The intestine, tail, and gills of tadpoles were isolated and immediately stored in RNAlater (Qiagen, Mississauga, Ontario, Canada) for analysis of gene expression.

Cell Culture

XLT-15 cells were maintained as described previously (Yaoita & Nakajima, 1997). The cultured cells were treated with 10 nM T_3 for 0, 2, 4, 8, 16, and 24 h. After the TH-treatment, total RNA was prepared from these cells.

Real time reverse transcription-polymerase chain reaction

The quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed by using a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) as described previously (Fujimoto *et al.*, 2006). DNase-treated

RNAs extracted from tadpole organs and cultured cells were used to prepare random-primed cDNAs with PowerScript (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocols. Real-time PCR reactions were carried out using SYBR Premix ExTaq (TaKaRa, Otsu, Japan). The levels of specific mRNA were quantified and normalized to the levels of *X. laevis* elongation factor 1-α (EF) (Krieg *et al.*, 1989), because its expression levels are unchanged during the spontaneous metamorphosis and in TH-treated XLT-15 cultured cells (Fujimoto *et al.*, 2006). Triplicate data obtained for each point were averaged. The primer sequences for the amplifications are described in Table 1.

PCR cloning of the 5'-ends of MMP and FAPα mRNAs

The transcription start site was determined by 5'-rapid amplification of cDNA ends (5'-RACE) (Frohman *et al.*, 1988). Reverse transcription was performed using 2.6 μg of total RNA from NF stage 63 *X. tropicalis* tadpole tails and PowerScript with the following primers: 5'-GCATCCTTAAGAACCATCA-3' for MMP-2, 5'-CATCGAAGGGGTAAGGATC-3' for MMP-9TH, 5'-TCTTGTCCCAGCGTCCTC-3' for MMP-11, 5'-CTCCTTCAGCTTGGTCTC-3' for MMP-13, and 5'-CTGAATATTTGGTGGAAGCTC-3' for FAPα. The 5' fragments of each cDNA were obtained using an anchor primer 5'-GTCGACATCGATCTCGAG(T)$_{18}$-3' and amplification primers as follows: 5'-GACATTTATCCTTAGGGCAG-3' for MMP-2, 5'-GGAATAGTTCAGGATACGA-3' for MMP-9TH, 5'-TGACGATTTCGCCCGCTG-3' for MMP-11,

5’-CCGGAATTCTGTAGGTCTCCTGGTGTC-3’ for MMP-13, and 5’-GCTGTGTAAGAGTATCTCC-3’ for FAPα. PCR products were cloned using the pGEM-T easy Vector Systems (Promega, Madison, WI, USA). Individual clones were isolated and sequenced using Thermo Sequenase Cycle Sequencing kit (Amersham, Buckinghamshire, UK).

Gel mobility shift assay

The gel mobility shift assay was performed as described previously (Fujimoto *et al.*, 2006). Briefly, *Xenopus* TRα and RXRα proteins were synthesized *in vitro* using the TNT kit (Promega). The sequences for the TRE binding experiments of one strand of nucleotides are follows: 5’-CCTAGGC**AGGTCA**TTTC**AGGACA**GCCCAGC-3’ for *Xenopus* TRβ DR4 TRE, 5’-CCTAGGC**AGGTCA**TTTC**A<u>AA</u>ACA**GCCCAGC-3’ for *Xenopus* TRβ mDR4 TRE (the nucleotides changed from wild type are underlined), 5’-TGTTTCC**TGTCCT**ACTC**TGCCCT**GCCTGTG-3’ for XtMMP-2 TRE1, 5’-ACTGTAC**TGTCCA**AGTC**TGACCC**GCACGAA-3’ for XtMMP-2 TRE2, 5’-TGTCTTC**TGCCCT**ACTC**TACCCT**GCCTGTG-3’ for XtMMP-2 TRE3, 5’-GAATCAT**TTACCT**CCTG**TAACCC**TGAGGAG-3’ for XtMMP-9TH TRE1, 5’-GAGTGTG**TGTCCT**TATC**TGACCT**CCAGTCA-3’ for XtMMP-9TH TRE2, 5’-ACTATCC**TGTCCT**TAGT**TTACCT**GCTATCC-3’ for XtMMP-9TH TRE3, 5’-AATTGTG**TGACCT**TACC**AGACCT**GCCATCA-3’ for XtMMP-11TRE1, 5’-AGCCTGA**AGGTCA**GTTA**AGGTGA**GATTGGA-3’ for XtMMP-11TRE2, 5’-CCATGGA**TGATCT**TATT**TGCCCT**ATTAATA-3’ for XtMMP-13 TRE1, 5’-CACATAC**AGACCC**ATAC**TGACCT**ATCTATC-3’ for XtMMP-13 TRE2,

5’-CACATAC**AGACCC**ACAC**TGACCT**ATCTATC-3’ for XtMMP-13 TRE3, 5’-CACATAC**AGACCC**AGAC**TGACCT**ATCTATC-3’ for XtMMP-13 TRE4, 5’-CTTACCA**TGCCCT**CCTG**TGACCC**CATTTAG-3’ for XtMMP-14 TRE1, 5’-CCTATAA**TGACTT**GTAA**TGATCC**CAACTGG-3’ for XtMMP-18 TRE1, and 5’-CTCTTCC**ATGTCA**AAGC**AGGTCA**TGGGGGG-3’ for XtFAPα TRE1. Bold letters indicate the TRE half-sites. Oligonucleotides were annealed by heating at 95°C for 5 min and then cooling to the room temperature before use as probes. The probes were labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (Amersham).

Results

Developmental expression of MMP mRNAs in the intestine, tail, and gills during the spontaneous metamorphosis

To compare the developmental expression profiles of MMP, FAPα and TRβ genes, quantitative RT-PCR was performed using total RNA prepared from the intestine, tail, and gills of *X. laevis* tadpoles from NF stage 57 to 63 during the spontaneous metamorphosis (Fig. 1). Gills were only analyzed from NF stage 57 to 61 due to the limited amount of tissue available. These three organs were chosen because they represent the tissue remodeling and resorption (Dodd & Dodd, 1976).

In the intestine, MMP-9TH, MMP-11, and MMP-14 mRNAs were expressed at low levels at NF stage 57, increased slightly at NF stage 58, showed the prominent rise at NF stage 60, and peaked at NF stage 61 (Fig. 1A) following the increase of endogenous TH (Leloup & Buscaglia, 1977). Thereafter, these expressions were decreased. These expression patterns resemble that of TRβ gene, although TRβ gene expression was much lower than mRNAs of the MMP genes (Fig. 1A). MMP-13 and FAPα were also up-regulated at NF stage 58 and had a very small peak at NF stage 60-61 (Fig. 1A). The developmental expression of these genes is roughly in parallel with the changes of T_3 concentrations in plasma (Leloup & Buscaglia, 1977). In contrast, MMP-2 gene increased to the maximum at NF stage 62 when adult epithelial cells proliferate. The expression of MMP-18 was very low and reached a very small peak at NF stage 62, consistent with the Northern blot analysis (Stolow *et al.*, 1996). The distinct expression patterns of MMP genes suggest that different ECM-degrading

proteinases play different roles in intestinal remodeling.

In the tail, MMP-9TH and MMP-14 genes were barely increased before NF stage 61, very strongly up-regulated at NF stage 62 when the tail starts rapid shortening (Nieuwkoop & Faber, 1956), and continued to increase till NF stage 63 (Fig. 1B). The expression levels were much higher than those in the intestine. Expression levels of MMP-9TH and MMP-14 mRNAs exceeded that of the elongation factor 1-α in copy number at NF stage 63. The expression of MMP-13, FAPα, and MMP-2 genes started to increase at NF stage 58, and was promoted abruptly at NF stage 62 concomitantly with MMP-9TH, MMP-11, and MMP-14 genes. TRβ mRNA was also up-regulated similarly to MMP-9TH and MMP-14 genes.

The up-regulation of MMP and FAPα genes was barely detectable from the beginning of the climax (NF stage 58) in gills. The drastic and coincident up-regulation was observed at NF stage 60-61 when the degradation of the branchial arches begins (Fig. 1C). The up-regulation of MMP-9TH, MMP-14 and MMP-13 genes was conspicuous at NF stage 61. TRβ expression was gradually increased from NF stage 58 to NF stage 61.

Expression of MMP mRNAs in the intestine, tail, and gills during the TH-induced metamorphosis

Since significant changes in the abundance of expressed transcripts for MMP and FAPα genes were shown in three organs of tadpoles undergoing the spontaneous metamorphosis, we examined the kinetics of MMP mRNA up-regulation during the TH-induced metamorphosis. The premetamorphic tadpoles are functionally athyroid but

competent to respond to exogenous TH (Dodd & Dodd, 1976). The addition of T_3 results in the activation of the *Xenopus* TRβ gene (Yaoita & Brown, 1990). To investigate the expression of MMP and FAPα genes during the T_3-induced metamorphosis, we treated the premetamorphic tadpoles immersed in 1 mM methimazole with 100 nM T_3 for 0-24 h or with 5 nM T_3 for 0-7 days. Total RNA was isolated from the intestine, tail and gills and analyzed by quantitative RT-PCR.

Addition of T_3 caused the significant increase in TRβ mRNA levels in three organs (Fig. 2A-C). TRβ expression was up-regulated by 6 h, and increasing slowly to higher levels after 12-24 h of treatment, consistent with our previous report (Yaoita & Brown, 1990). In contrast to TRβ, the significant induction of MMP-9TH, MMP-11, MMP-13, MMP-14, and FAPα mRNAs was observed after 12-24 h of treatment (Fig. 2A-C). The expression of MMP-14, MMP-9TH, and MMP-11 genes increased obviously in the intestine, while MMP-14, MMP-13 and FAPα genes were up-regulated in the tail and gills. These five genes were activated to low levels 6 h after T_3 treatment. The expression of MMP-2 and MMP-18 genes increased a little within 24 h, and was up-regulated more in the tail after the extended T_3 treatment (Fig. 2D). Most of MMP and FAPα genes reproduced developmental gene expression profiles in three organs. The detailed analysis indicates that mRNA copy numbers of TH-responsive genes in three organs of 24 h-treated tadpoles are comparable to those seen at NF stage 59-60 during the spontaneous metamorphosis (Fig. 1,2 and data not shown).

To confirm the T_3–induction of MMP and FAPα mRNAs in a myoblastic cell line derived from a tadpole tail, quantitative RT-PCR was performed using RNAs

extracted from XLT-15 cells stimulated with 10 nM T_3 (Fig. 3). TRβ mRNA started to increase 8 h after T_3 treatment of XLT-15 cells and then slowly (Fig. 3), consistent with the up-regulation by 8 h in T_3-treated XL-177 cells derived from embryonic epithelial cells (Kanamori & Brown, 1992). The elevation in MMP-9TH mRNA began soon after TH treatment, and the great parts of the up-regulation were observed in 16-24 h in agreement with our results (Fujimoto *et al.*, 2006). The expression of MMP-14 and MMP-11 genes was also activated just after TH stimulation. MMP-13 and FAPα mRNAs increased by 4-8 h, but their prominent up-regulation was observed after 2-4 days of exposure to 10 nM T_3 (data not shown). The expression of MMP-2 and MMP-18 was changed little by T_3 treatment.

Identification of TREs in MMP and FAPα Genes

It has been shown that MMP-9TH and MMP-11 genes of *X. laevis* were direct TH- response genes, *i.e.,* activated at the transcriptional level through the nuclear receptors of TH (Fujimoto *et al.*, 2006, Fu *et al.*, 2006), whereas MMP-2, MMP-13, MMP-14, MMP-18, and FAPα genes are suggested to be late indirect T_3 response genes (Wang & Brown, 1993, Stolow *et al.*, 1996, Hasebe *et al.*, 2006). Since MMP-13 and FAPα genes were up-regulated like MMP-9TH and MMP-11 genes in the tail and gills of T_3-treated tadpoles, we investigated whether there are any thyroid hormone response elements (TREs) in the promoters of MMP and FAPα genes.

To determine the transcription start site of *X. tropicalis* MMP and FAPα genes, 5'-RACE was carried out using total RNA prepared from NF stage 63 tadpole tails of *X. tropicalis*. Several MMP-9TH and MMP-13 cDNA clones were obtained that showed

clearly the position of the transcription start site and that had only a few nucleotide changes compared with their respective genomic sequences. These changes were likely derived from sequence polymorphisms or PCR errors by Taq polymerase. A consensus TATA box was found 23-25 bp upstream of the start sites of both genes. In contrast, the sequence analysis of MMP-2, MMP-11, and FAPα clones suggested the existence of more than one start site. No TATA box was present upstream of the 5'-ends of MMP-2 and FAPα clones, implying that these genes have a TATA-less promoter. Although a putative TATA box was found in MMP-11 promoter, it might not be effective as previously shown in *X. laevis* (Li *et al.*, 1998). The full-length cDNAs of MMP-14 and MMP-18 were found in the *X. tropicalis* database.

The genomic structures of *X. tropicalis* MMP and FAPα genes were determined by analyzing the *X. tropicalis* genome database (DOE Joint Genome Institute). We searched for putative TREs in MMP and FAPα genes by using the NHR Scan (Sandelin & Wasserman, 2005). In MMP-9TH and MMP-11 genes, the relative positions of putative TREs are conserved between *X. laevis* and *X. tropicalis*, which might indicate a functional role of these elements.

To assess whether TR/RXR heterodimer can bind to the putative TREs found in MMP and FAPα genes, the gel shift assay was performed on elements in their natural context (16 bp core with 7 bp of flanking sequences) using *in vitro* synthesized TR and RXR. The high-affinity TRE are described as a perfect direct repeat of AGGTCA separated by 4 bp (DR4) (Umesono *et al.*, 1991). The binding of TR/RXR complexes to a labeled MMP TRE was competed with the oligonucleotide containing the wild type or

mutated version of TRE derived from the TRβ promoter (Ranjan *et al.*, 1994) (Table 2, Fig. 4). Figure 5 shows the positions of putative TREs lying between -10 kb and +10 kb of the transcriptional start site of each gene. All of the end-labeled TREs failed to form any specific complexes with the TR or RXR alone. The gel mobility shift assay revealed that TR/RXR heterodimer binds to the DR4 of MMP-9TH, MMP-11, and MMP-14 strongly, whereas MMP-2 and MMP-18 TREs form a complex weakly (Table 2, Fig. 4,5). MMP-13 and FAPα TREs showed the moderate affinity to TR/RXR heterodimer. No significant binding was detected with the DR4 of MMP-2 TRE2, MMP-2 TRE3, MMP-13 TRE1, and MMP-13 TRE2 (Table 2). MMP-9TH and MMP-11 TREs are a close match to the DR4 of optimal TRE, while MMP-18 TRE1 is more divergent (Table 2). These bindings were competed by 50-fold excess of cold TRβ DR4, but not by the same amount of mutated version of TRβ DR4. Since the G residues at the second and third positions of the second half-site are absolutely required for binding (Kurokawa *et al.*, 1993), our results suggest that TREs capable of binding to TR/RXR heterodimer *in vitro* are functional in the transcriptional regulation of MMP genes.

Discussion

Cells die in the regressing organs by two mechanisms, suicide and murder (Nakajima *et al.*, 2005). A myoblastic cell line, XLT-15, derived from a tadpole tail responds to TH by apoptosis (Yaoita & Nakajima, 1997), indicating that tail muscle cells die cell-autonomously (a suicide model). The up-regulation of MMP-11 and MMP-13 in the myotendinous junctions of a regressing tail implies that the TH-induced secretion of MMP results in the degradation of the myotendinous junctions, which detaches muscle cells from ECM and causes their death (a murder model) (Berry *et al.*, 1998b). We showed previously that TH induces the suicide of muscle cells at the beginning of the climax, and then both the murder and suicide in a regressing tail to execute the death of all muscle cells (Nakajima & Yaoita, 2003). Since ECM-degrading proteinases are believed to play an important role in murder mechanism by regulating cell-cell interactions, we have examined the developmental expression profiles of MMP genes and their induction in the TH-treated tadpoles and cultured cells in order to understand the molecular mechanism of the TH-induced MMP gene expression. The gel mobility shift assay was also carried out to address whether the heterodimer of TR and RXR can bind to the putative TREs that are speculated by the sequence analogy in the *X. tropicalis* MMP genes.

The regulation of MMP gene expression during the climax of metamorphosis

Several reports show that MMP gene expression is induced in morphologically changing organs during the climax of metamorphosis, as mentioned above. The *in situ* hybridization analysis shows that FAPα, MMP-11, MMP-13, MMP-14, and MMP-2

genes are expressed in connective tissue, subepidermal fibroblast, notochord sheath, notochord, myoseptum, and spinal cord of a regressing tail (NF stage 62-63), and that MMP-11, MMP-13, MMP-14, and MMP-2 mRNAs are located in connective tissue of the remodeling intestine, although there are some differences in signal intensities (Ishizuya-Oka *et al.*, 1996, Berry *et al.*, 1998b, Damjanovski *et al.*, 1999, Jung *et al.*, 2002, Hasebe *et al.*, 2006). A comprehensive expression study using the common materials is essential to understand the profiles of all TH-induced MMP genes and to know the molecular mechanism of their expression. We have showed that the expression of MMP genes is induced abruptly and concurrently in the different organs at the different stages when the morphological changes take place during the climax of metamorphosis.

In the intestine, MMP-14, MMP-11, and MMP-9TH gene expression starts prominently at NF stage 60, and rises in parallel with the endogenous T_3 concentration, which has a peak around NF stage 61. The expression of FAPα and MMP-13 genes also peak at the similar stage, although their maximal expression is quite low compared with MMP-14, MMP-11, and MMP-9TH genes. The expression profiles of these five genes might reflect the T_3 concentration in plasma as TH-responsive genes. Because the intestine expresses a low level of type III deiodinase known to inactivate active TH (T_3) till NF stage 61 (data not shown), which makes this organ sensitive to TH (Becker *et al.*, 1997). On the other hand, the expression of MMP-2 gene is maximal at NF stage 62. This expression might be due to the secondary response to the apoptotic degeneration of the larval epithelial cells and the proliferation of the adult epithelial cells.

ECM-degrading proteinase genes including FAPα are induced dramatically and simultaneously at NF stage 62 and continue to increase in a regressing tail. The induction of MMP gene expression in the tail is clearly delayed compared with the intestine. This time lag might be caused by a high-level expression of type III deiodinase from NF stage 59 to 61 in the tail (Wang & Brown, 1993), since type III deiodinase regulates elaborately the cell proliferation and innervation during the development of retina by inactivating T_3 (Marsh-Armstrong *et al.*, 1999), and can inhibit the anuran metamorphosis, when this gene is overexpressed in a transgenic tadpole (Huang *et al.*, 1999). However, why type III deiodinase, one of direct TH-response genes, is down-regulated at NF stage 62 when a level of T_3 is still high in plasma remains to be elucidated (Kawahara *et al.*, 1999).

The concomitant and conspicuous induction of MMP genes begins at NF stage 60-61 in gills, which indicates MMP gene expression is repressed at NF stage 58 to 59 when T_3 is increasing. Gills might not be sensitive enough to respond to a low level of T_3 by the unknown reasons, compared with the intestine.

MMP-9TH and MMP-14 among the examined genes are prominently co-expressed in the remodeling intestine and the regressing tail and gills, suggesting that they play an important role as main effectors in transforming organs. MMP-9TH has a strong gelatinolytic activity (Fujimoto *et al.*, 2006), and degrade native type IV collagen (Wilhelm *et al.*, 1989) that is the major structural component in the basement membrane. MMP-11 and MMP-13 are also strongly induced in remodeling intestine and regressing organs (a tail and gills), respectively. MMP-11 and the membrane type MMP such as

MMP-14 are activated by a furin-dependent cleavage intracellularly (Pei & Weiss, 1995, Vu & Werb, 2000), but the other MMP proteins are secreted as inactive proenzymes and their activation requires the removal of propeptide by cleavage, which imply that MMP-14 and MMP-11 can contribute to the morphological changes as initiator proteinases. MMP-14 can activate MMP-2 and MMP-13 on the cell surface (Itoh & Seiki, 2006). It is suggested that MMP-14 participates in an activation cascade involving MMP-13, MMP-2, and MMP-9 (Cowell *et al.*, 1998). It is possible that the intracellularly activated MMP-14 increases in transforming organs, and processes the other TH-induced MMP proenzymes proteolytically into their biologically active forms to degrade ECM, and that activated MMP-9TH digests the basement membrane, which underlies all epithelial cell sheets in the intestine and gills and surrounds individual muscle cells and notochord in the tail.

A function-blocking antibody against MMP-11 inhibits TH-induced apoptosis of the larval epithelium, thickening of the basement membrane, and the invasion of the adult epithelial primordia into the connective tissue in an organ culture of the intestine (Ishizuya-Oka *et al.*, 2000). The precocious expression of MMP-11 in the intestine leads to ECM degradation and larval epithelial cell death in transgenic tadpoles expressing MMP-11 under control of a heat shock-inducible promoter (Fu *et al.*, 2005). Moreover, MMP-11 is one of the early-induced genes in the intestine during the climax. It is possible that the TH-induction of the intracellularly activated MMP-11 triggers the ECM remodeling in the intestine, although there are no reports about MMP activation by MMP-11 to our knowledge.

The two steps of MMP gene induction by TH

When methimazole-treated tadpoles (NF stage 54) are stimulated by 100 nM T_3, TRβ gene expression is up-regulated within 6 h in three organs and slowly increased at 16-24 h. On the other hand, small amounts of MMP-9TH, MMP-11, MMP-13, MMP-14, and FAPα mRNAs are induced just after T_3 treatment, and their great parts of TH-induction are observed after 12 h of treatment. In T_3-treated cultured cells, TRβ mRNA expression starts within 8 h, and is saturated around a day of treatment, while the up-regulation of MMP-9TH, MMP-11, MMP-13, MMP-14, and FAPα genes are accelerated after 16-24 h of exposure to 10 nM T_3. Especially MMP-9TH mRNA is up-regulated more than 15 times within 2 h. In all cases, the expression of MMP genes are induced to very low levels just after T_3 treatment, and increase prominently 12 h later, whereas TRβ gene is activated in several hours, and up-regulated slowly after 16 h of treatment. The time course of TRβ gene expression resembles that of the directly TH-induced transcriptional factor, that is, the zinc finger transcription factor xBTEB1 (Furlow & Brown, 1999).

We and the other group reported the presence of the functional TREs in the promoters of *X. laevis* MMP-9TH and MMP-11 genes, respectively (Fujimoto *et al.*, 2006, Fu *et al.*, 2006). Our gel mobility shift assay has revealed that all TH-induced ECM-degrading proteinase genes have TREs in *X. tropicalis*. The strongly and early induced genes such as MMP-14, MMP-11, and MMP-9TH have higher affinity TREs, while the weakly and late induced genes like MMP-2 and MMP-18 contain a lower affinity TRE (Furlow & Kanamori, 2002). Moderate affinity TREs are located in the

promoters of MMP-13 and FAPα genes that are expressed at higher levels in the regressing tail and gills, and induced reasonably in the tail and gills of TH-treated tadpoles.

We searched for the putative TREs in 10 Mb of scaffold 1 and 2 from *X. tropicalis* genome database using the NHR Scan, and found total 1053, of which 593, 417, 42, and 1 TREs share 9, 10, 11, and 12 of 12 nucleotides with an optimized TRE sequence (AGGTCAnnnnAGGTCA), respectively. 50% of TREs (9/12), 71% of TREs (10/12), and all TREs (11/12) showed a specific complex in the presence of TR/RXR in our gel mobility shift assay. The expected numbers of TREs that can bind to TR/RXR might be 0.19 and 1.27 in 3 kb and 20 kb genomic sequence, respectively. TREs are located between -0.8 kb and +1.5 kb of the transcription start site in MMP-9TH, MMP-11, MMP-14 and FAPα genes. MMP-13 gene has TREs 9 kb downstream of the transcription start site, although they are 4 copies of TREs (+) and one copy of TRE (++). MMP-2 and MMP-18 genes contain one TRE (+) 5.9 kb downstream and 9.4 kb upstream, respectively, and it is suspected that they have TRE incidentally due to a probability, but not inevitably. However, TRE in xBTEB1 gene lies 6.5 kb upstream of the transcription start site, and is demonstrated to be functional in the gel mobility shift assay and transient transfection assay (Furlow & Kanamori, 2002). This position of TRE is also conserved in *X. tropicalis* BTEB1 gene. It is necessary to compare the promoter sequences between *X. tropicalis* and *X. laevis* and to confirm whether these TREs are functional or not by the chromatin immunoprecipitation assay. We have shown that MMP-13, FAPα, and MMP-2 mRNAs increase in the tail at NF stage 58-61, whereas

MMP-9TH and MMP-11genes are suppressed. Since MMP-13, FAPα, and MMP-2 genes have no high affinity TREs, they are weakly repressed by TR and activated easily by some developmentally up-regulated transcriptional factors, while MMP-9TH and MMP-11 genes with high affinity TREs are depressed by unliganded TR in reduced levels of T_3 by type III deiodinase. The micro array analysis for 21654 genes of *X. laevis* reveals that approximately 30% of examined genes are up- or down-regulated by TH (Das *et al.*, 2006), raising a possibility that most of them are controlled more or less directly through TREs.

These results lead us to propose the two steps of MMP gene induction by TH. As a level of T_3 is rising, T_3 is forming a complex with TRα/RXR already bound to TRE in the promoters of TRβ, other TH-induced transcriptional factor, and MMP genes, and directly induces their expression to low levels. TH-induced transcriptional factors including TRβ protein accumulate and cause the second strong activation of MMP genes. A high affinity TRE can form a stable complex with TR/RXR heterodimer, and directs an early response, whereas a lower affinity TRE such as those of MMP-13 and FAPα genes cannot bind firmly and delays a TH-response. A very low affinity TRE (+) in MMP-2 and MMP-18 genes only give a marginal effect of TH to their promoter, and their expression is dependent mainly on the gradually-induced transcriptional factors by TH.

The up-regulation of TRβ mRNA in the tail and head results in the increase of TR proteins (Eliceiri & Brown, 1994), which should promote the binding of TR to TRE in MMP genes and activate their transcription in the presence of TH. Some TH-induced

transcriptional factors might be coactivators of TR, since steroid receptor coactivator 3 (SRC3) mRNA and protein increase in both the tail and intestine during the natural as well as TH-induced metamorphosis, and this protein is recruited to target genes by TR (Paul *et al.*, 2005). The induced SRC3 is expected to activate MMP genes strongly through binding to TR on their TRE in concert with TH-induced TRβ.

However, some factors do not appear to be coactivators. It is because TRβ mRNA increases gradually and linearly in gills from NF stage 58, whereas MMP genes show the drastic induction at NF stage 60. The expression of more than 360 transcriptional factor genes are known to be regulated by TH (Das *et al.*, 2006), suggesting a possibility that some of them contribute to the synchronous and abrupt induction of MMP genes in the different organs at the different stages.

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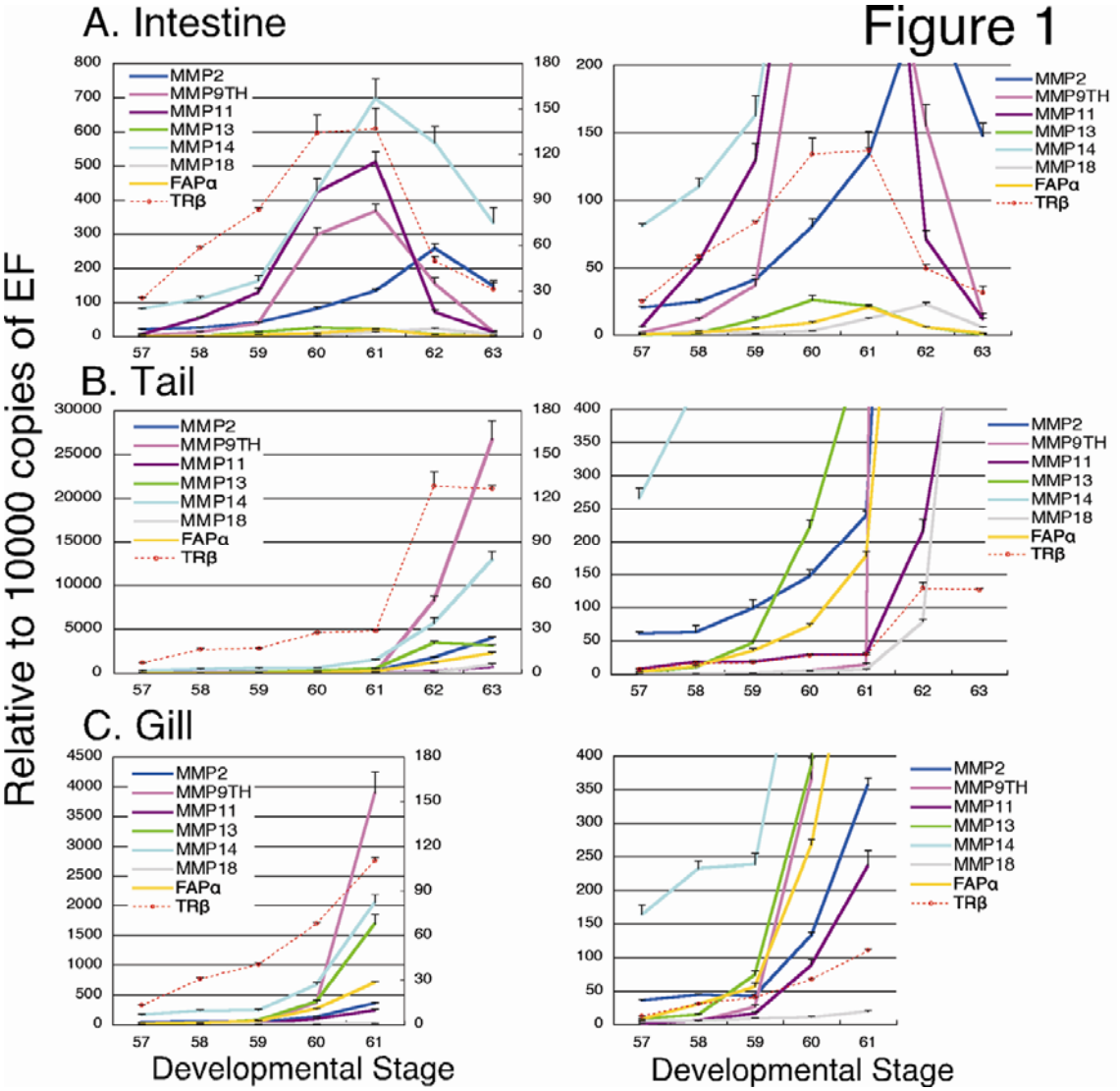

Figure 1. Developmental expression of MMP, FAPα, and TRβ genes in the intestine, tail, and gills during the spontaneous metamorphosis.

Total RNA was extracted from the intestine, tail and gills of *X. laevis* tadpoles at indicated developmental stages and subjected to quantitative RT-PCR to determine MMP, FAPα, and TRβ mRNA levels. Real-time RT-PCR was performed using total RNA prepared from the intestine (A) and tail (B) of NF stage 57-63, and gills (C) of NF stage 57-61 tadpoles. Each point was performed three times. The error bars represent the SE. The levels of MMP, FAPα, and TRβ mRNAs are shown as copy numbers

relative to 10,000 copies of EF mRNA. Note for TRβ, the scale of the vertical axis is indicated at the right side of each left panel.

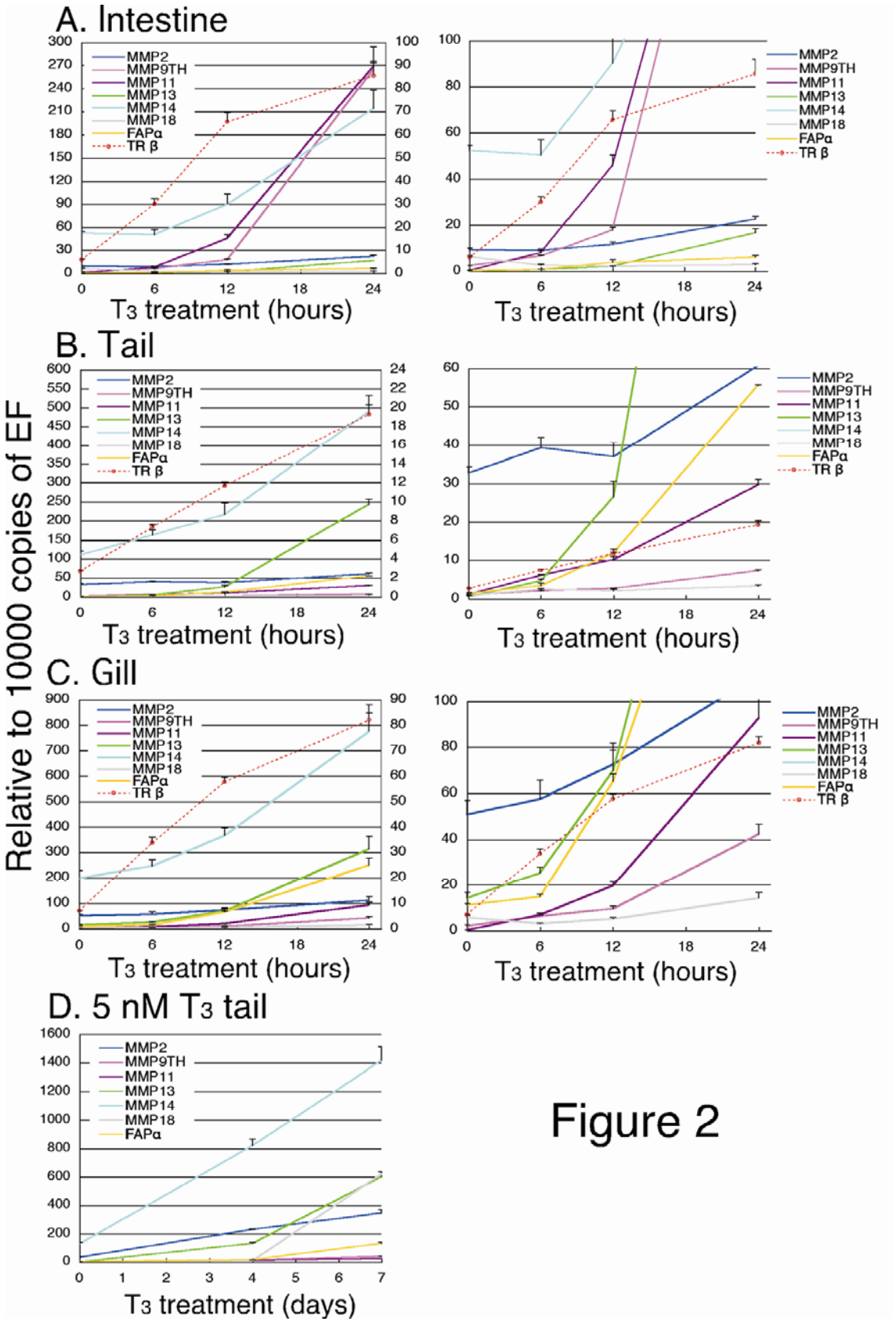
A. Intestine
B. Tail
C. Gill
D. 5 nM T3 tail
Relative to 10000 copies of EF
MMP2
MMP9TH
MMP11
MMP13
MMP14
MMP18
FAPα
TR β
T3 treatment (hours)
T3 treatment (days)

Figure 2

Figure 2. T_3-induced expression of MMP, FAPα, and TRβ mRNAs in tadpole organs.

Quantitative RT-PCR was performed to assess the time course of T_3-induced up-regulation of MMP, FAPα, and TRβ genes in tadpole organs. Total RNA was prepared from the intestine (A), tail (B), and gills (C) in *X. laevis* premetamorphic tadpoles treated with 100 nM T_3, and the tail (D) in tadpoles treated with 5 nM T_3 in the presence of 1 mM methimazole for the indicated times. Each point was performed at least three times. The error bars represent the SE. The levels of MMP, FAPα, and TRβ mRNAs are shown as copy numbers relative to 10,000 copies of EF mRNA. Note for TRβ, the scale of the vertical axis is indicated at the right side of each left panel.

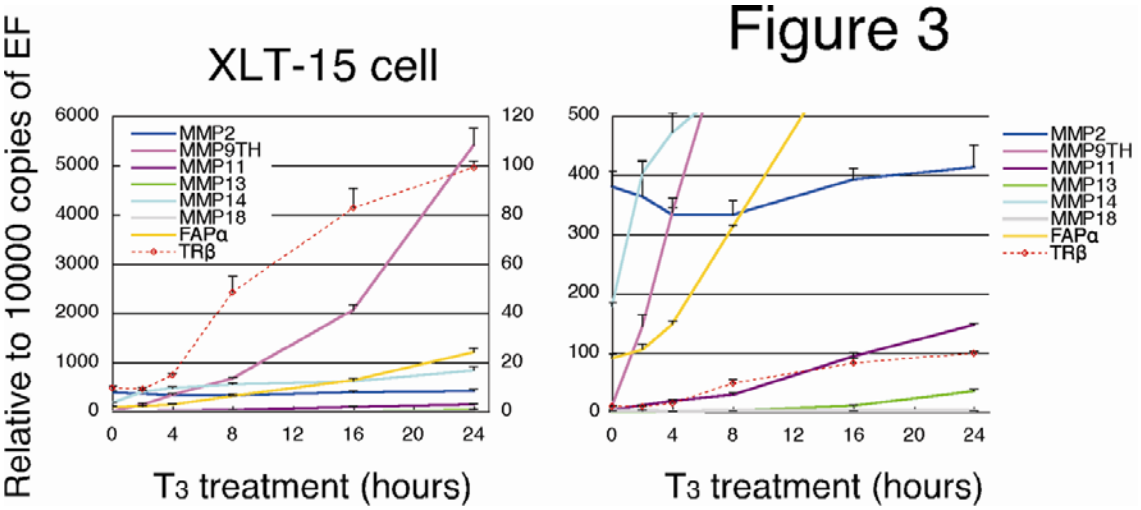

Figure 3. T3-induced expression of MMP, FAPα, and TRβ mRNAs in XLT-15 cultured cell line.

Quantitative RT-PCR was performed to assess the time course of T3-induced up-regulation of MMP, FAPα, and TRβ genes in XLT-15 cells. Total RNA was extracted from XLT-15 cells that were cultured in the presence of 10 nM T3. Each point was performed three times. The error bars represent the SE. The levels of MMP, FAPα, and TRβ mRNAs are shown as copy numbers relative to 10,000 copies of EF mRNA. Note for TRβ, the scale of the vertical axis is indicated at the right side of the left panel.

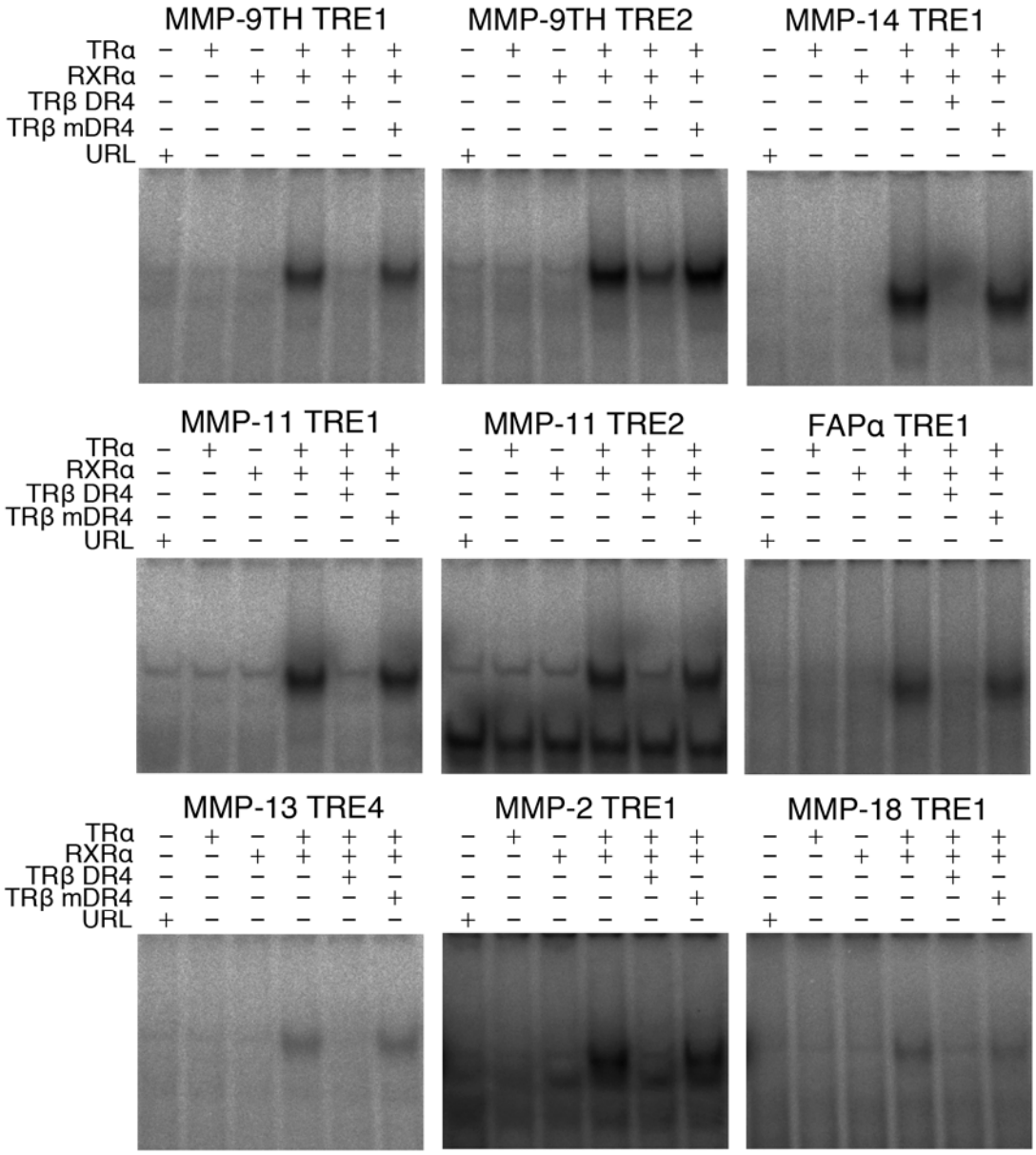

Figure 4. The binding of MMP and FAPα TREs to *in vitro* translated TR and RXR.

The gel mobility shift assay was carried out using the end-labeled probes that are indicated at the top of each panel. A labeled DR4 element was incubated in the presence of unprogrammed lysate (URL) or TR or/and RXR with or without a 50-fold excess of the indicated non-radioactive competitor oligonucleotide. TRβ DR4 and TRβ mDR4 are wild type and mutant TREs derived from the T_3-regulated TRβ gene

promoter, respectively (see Table 2). The gels were exposed for the following times: MMP-9TH TRE1 and TRE2, MMP-14 TRE1, MMP-11 TRE1 and TRE2, FAPα TRE1, and MMP-13 TRE4, 1 h; MMP-2 TRE1 and MMP-18 TRE1, 16 h.

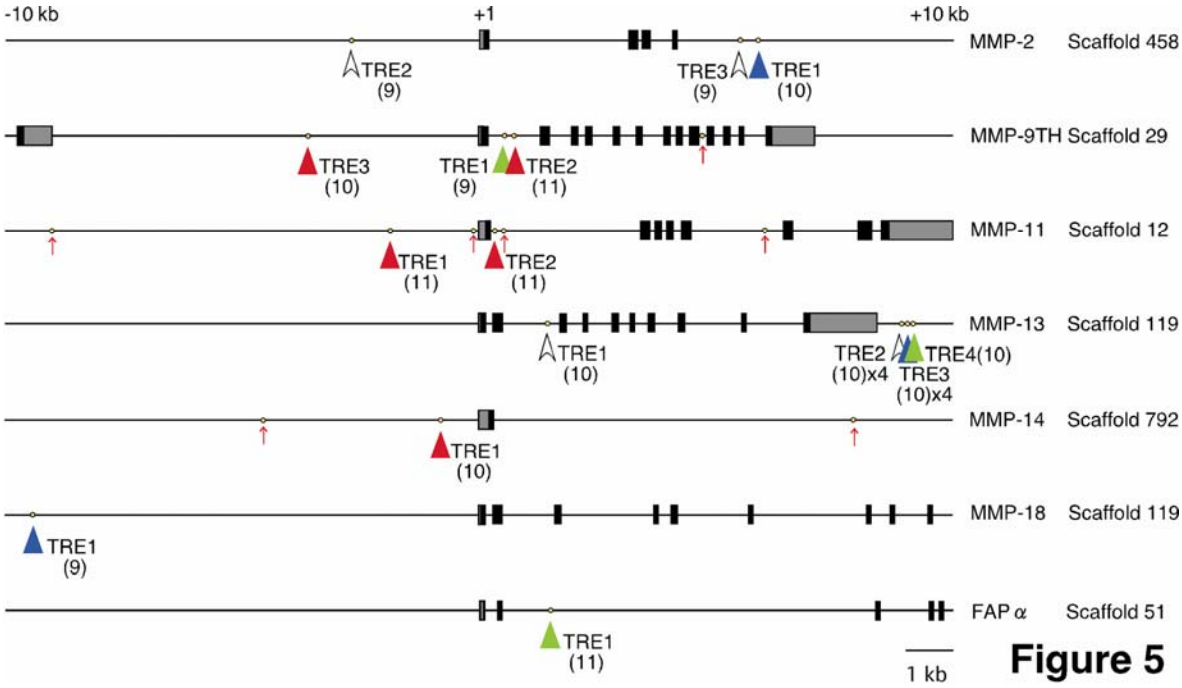

Figure 5. Distribution of TREs in MMP and FAPα genomic genes of *X. tropicalis*.

The genomic structures of *X. tropicalis* MMP and FAPα genes were conducted from *X. tropicalis* genome database. The boxes represent exons. The black and gray boxes indicate the putative protein coding and untranslated regions, respectively. The distribution of TREs was identified by NHR Scan between -10 kb and +10 kb of the transcription start site (+1) of each gene. MMP-9 gene is located 9 kb upstream of MMP-9TH gene on the chromosome in the same transcriptional orientation (Fujimoto *et al.*, 2006). The small red arrows show the putative TREs (over 9/12) that were not analyzed in this study, while the arrowheads mean TREs that were used in the gel shift assay (see Fig. 4). The white arrowheads imply the inability to form a complex, and the blue and green arrowheads show the weak and strong ability to form a complex in the presence of TR/RXR, respectively. The red arrowheads correspond to the strongest ability. The identity with DR4 (AGGTCAnnnnAGGTCA) is shown in

parenthesis.

Table 1. Primers used in quantitative PCR.

Primers	Sequence (5’ to 3’)
XlMMP2-FW2	TGGTAATGCAGATGGAGAGTTC
XlMMP2-RV2	AGTTAAGGTAAATAGCAACTCATGTG
XlMMP9TH-FW2	CTCTCAGCCAAATGCAAAGT
XlMMP9TH-RV2	GTTTAGGATACGATATGTGAG
XlMMP11-FW1	CTGACGTACAAGATCATCCG
XlMMP11-RV1	TTATCTCCATGCCAGTACCG
XlMMP13-FW2	CTCTTTGTTGTTGCTGCTC
XlMMP13-RV2	AGGACCATACAGAACCTGAA
XlMMP14-FW4	AAGGAGCATTCATGGGCAGTGATG
XlMMP14-RV6	CCATCCAGTCGACCAAAACGGA
XlMMP18-FW1	AGGAAGAAATGGCAGAGAAT
XlMMP18-RV1	GTCCAATGTTCCTGTCACC
XlFAP-FW1	GTTGTAACTCTGCTAATAGTTACTGT
XlFAP-RV1	CGGATATATTCGTTCTCTGAAATCCA
XlTRβ-FW1	GAATGGCAACAGACTTGGTT
XlTRβ-RV1	GCTTCAGTGACAACTTGTATC

Table 2. TRE sequences in the *X. tropicalis* MMP and FAPα genes.

In the optimized sequence, 'n' means any nucleotide.

TRE sequences of MMP and FAPα genes are aligned with an optimized TRE sequence. The AGGTCA-like motifs of the DR4 are in the capital letters, and bold nucleotides indicate identity with the consensus motif. The intervening letters are in lowercase. The plus and minus signs mean the ability and inability to form a specific complex in the presence of TR/RXR in the gel shift assay, respectively. The number of plus signs corresponds to the intensity of an observed complex: a single plus shows the weak ability to form a complex (for example see Fig. 4 for MMP-2 and MMP-18 TREs) and three plus signs correspond to the strongest ability to form a complex (see Fig. 4 for MMP-9TH and MMP-11 TREs).

Gene	TRE	Relative binding of TR
DR4 TRE	**AGGTCA**nnnn**AGGTCA**	
xTRβ DR4	**AGGTCA**tttc**AGG**A**CA**	
xTRβ mDR4	**AGGTCA**tttc**AAA**A**CA**	
XtMMP-2 TRE1	**AGG**G**CA**gagt**AGG**A**CA**	**+**
XtMMP-2 TRE2	G**GGTCA**gactT**GG**A**CA**	**-**
XtMMP-2 TRE3	**AGG**GT**A**gagt**AGG**G**CA**	**-**
XtMMP-9TH TRE1	G**GGT**T**A**cagg**AGGTA**A	**++**
XtMMP-9TH TRE2	**AGGTCA**gata**AGG**A**CA**	**+++**
XtMMP-9TH TRE3	**AGGTA**A**C**acta**AGG**A**CA**	**+++**
XtMMP-11 TRE1	**AGGTC**Tggta**AGGTCA**	**+++**

XtMMP-11 TRE2	**AGGTCA**gtta**AGGT**G**A**	**+++**
XtMMP-13 TRE1	**AGG**G**CA**aata**AG**A**TCA**	**-**
XtMMP-13 TRE2	**AGGTCA**gtatG**GGTC**T	-
XtMMP-13 TRE3	**AGGTCA**gtgtG**GGTC**T	+
XtMMP-13 TRE4	**AGGTCA**gtctG**GGTC**T	++
XtMMP-14 TRE1	G**GGTCA**cagg**AGG**G**CA**	**+++**
XtMMP-18 TRE1	G**GATCA**ttac**AA****GTCA**	**+**
XtFAPα TRE1	**A**T**GTCA**aagc**AGGTCA**	**++**