One of the Duplicated Matrix Metalloproteinase-9 Genes is Expressed in Regressing Tail during Anuran Metamorphosis

Running Title: Gene duplication of Anuran MMP-9

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Abstract

The drastic morphological changes of tadpole are induced during the climax of anuran metamorphosis, when the concentration of endogenous thyroid hormone is maximal. The tadpole tail, which is twice as long as the body, shortens rapidly and disappears completely in several days. We isolated a cDNA clone, designated as Xl MMP-9TH, similar to the previously reported *Xenopus laevis* MMP-9 gene, and show that their *Xenopus tropicalis* counterparts are located tandemly about 9 kb apart from each other in the genome. *Xenopus* MMP-9TH gene was expressed in the regressing tail and gills and the remodeling intestine and central nervous system, and induced in thyroid hormone-treated tail-derived myoblastic cultured cells, while MMP-9 mRNA was detected in embryos. Three thyroid hormone response elements in the distal promoter and the first intron were involved in the up-regulation of Xl MMP-9TH gene by thyroid hormone in transient expression assays, and their relative positions are conserved between *Xenopus laevis* and *tropicalis* promoters. These data strongly suggest that MMP-9 gene was duplicated, and differentiated into two genes, one of which was specialized in a common ancestor of *Xenopus laevis* and *tropicalis* to be expressed in degenerating and remodeling organs as a response to thyroid hormone during metamorphosis.

Key words: Metamorphosis, Matrix metalloproteinase-9, Thyroid hormone, Programmed cell death, Gene duplication.

Introduction

The extracellular matrix (ECM) plays a critical role in organogenesis and tissue remodeling by serving as a structural support and a medium for cell-cell interactions. Matrix metalloproteinases (MMPs) constitute a family of proteolytic enzymes acting to degrade the components of ECM, and have been implicated in many physiological processes including the embryonic development, tissue resorption and remodeling, and pathological events such as tumor invasion and arthritis (Martel-Pelletier 1999; Nelson *et al.* 2000; Vu & Werb 2000). All MMPs have a similar domain structure with the pre-peptide to target for secretion, the pro-domain to maintain latency, and the catalytic domain that contains the zinc-binding active site. The pro-domain (about 80 amino acids) contains a highly conserved sequence, PRCGXPD. The cysteine within this sequence interacts with the catalytic zinc to maintain the enzyme in latent form (Van Wart & Birkedal-Hansen 1990). MMPs are produced as latent enzymes, and processed to an active form by the removal of the pro-domain through various mechanisms (Woessner 1991).

Among the previously reported MMPs, MMP-2 and MMP-9 are key enzymes that degrade native type IV collagen and gelatin (Okada *et al.* 1992; Wilhelm *et al.* 1989; Woessner 1991). Type IV collagen is the major structural component in the basal lamina that underlies all epithelial cell sheets and tubes and surrounds individual muscle cells, fat cells, and Schwann cells. These MMPs have three fibronectin-type II repeats inserted into the catalytic domain. These repeats interact with collagen and gelatin (Allan *et al.* 1995; Steffensen *et al.* 1995).

The dynamic changes from a larva to an adult are observed during the climax of anuran metamorphosis that is induced by a surge of thyroid hormone (TH) (Dodd & Dodd 1976; Nakajima *et al.* 2005). This systematic reorganization includes the absorption of larva-specific organs such as a tail and gills, the appearance of adult type organs like hind limbs, and the remodeling of intestine, pancreas, brain, skin, etc. In intestine, the larval epithelial cells undergo programmed cell death, and then replaced by the newly differentiated adult epithelial cells (Bonneville 1963). Although these metamorphic changes are caused by the increase of TH in plasma, the stages at which maximal cell death is observed are different in different organs. Metamorphosis can be precociously induced by TH

treatment of tadpole, and tissue resorption can be also reproduced when tadpole tail is stimulated by TH in the organ culture. MMP genes such as stromelysin-3, collagenase-3 (Brown *et al.* 1996), and collagenase-4 (Stolow *et al.* 1996) are induced in TH-treated tail, and expressed in regressing tail during the spontaneous metamorphosis. TH treatment of organ-cultured tails augments MMP-2 and MT3-MMP mRNA, and is suggested to increase a gelatinolytic activity by MMP-9 on gelatin zymogram (Jung *et al.* 2002).

Two mechanisms are proposed to explain cell death in regressing tail during the climax of metamorphosis. We have established a myoblastic cell line (XLT-15) derived from *Xenopus laevis* tadpole tail. This cultured cell line dies in response to TH, which means that tail muscle cells die cell-autonomously (a suicide model) (Yaoita & Nakajima 1997). Furthermore, this model is supported by the report that tail muscle cell death precociously induced by TH is inhibited by skeletal muscle-specific expression of dominant-negative thyroid hormone receptor (DNTR) gene in transgenic tadpoles (Das *et al.* 2002). On the other hand, ECM-degrading enzymes like stromelysin-3 and collagenase-3 are highly expressed in the tail subepidermal fibroblasts encircling the entire muscle flank but not in tail muscle. These enzymes are also up-regulated in the myotendinous junctions to which the muscle fibers are attached. These observations lead to the idea that the increase of secreted MMPs induced by TH results in the degradation of the myotendinous junctions, which detaches muscle cells from ECM and causes their death (a murder model) (Berry *et al.* 1998). When DNTR gene is introduced into muscle cells by the somatic gene transfer to block TH-signaling, DNTR-transfected muscle cells are protected from death before tail shortening, but partially in regressing tail during the spontaneous metamorphosis. This result means that TH induces the cell-autonomous death of tail muscle at the beginning of metamorphosis climax, and both the murder and suicide mechanisms to execute muscle cell death in regressing tail (Nakajima & Yaoita 2003).

It is known that the cascade of caspase activation leads to apoptosis. Caspase mRNAs increase in regressing tail and remodeling intestine (Nakajima *et al.* 2000; Rowe *et al.* 2002). Bax, a proapoptotic member of bcl-2 protein family, is implied to be a TH-inducible regulator of the cell-autonomous muscle fiber death in the regressing tail during metamorphosis (Sachs *et al.* 2004). In

addition, ECM-degrading enzymes is believed to play a pivotal role in murder mechanism by regulating cell-cell interactions and detachment of muscle cells from myotendinous junctions. In the experiment using transgenic tadpole, the stromelysin-3 gene expression is shown to induce larval epithelial cell death and fibroblast activation in intestine, events that normally occur only in the presence of TH, through ECM remodeling (Fu *et al.* 2005).

In this study, we have focused on MMP-9 which can cleave native type IV collagen. This collagen is distributed in the basal lamina between muscle cells and in the notochord lamella of tadpole tail (Nakajima & Yaoita 2003). We show that MMP-9 gene is duplicated in *Xenopus* genomes, but not in the other vertebrate genomes reported to date. One of *Xenopus* MMP-9 genes, MMP-9TH, was expressed strongly in regressing tail and remodeling intestine (the early phase) and central nervous system during the climax of anuran metamorphosis, and induced more than 280-fold in 3,3',5-triiodo-L-thyronine (T_3)-treated myoblastic cultured cells (XLT-15) derived from tadpole tail. The other MMP-9 gene originally reported was expressed in embryos (Carinato *et al.* 2000), and induced in the late phase of remodeling intestine, whereas its expression does not impressively increase in regressing tail, remodeling central nervous system, and T_3-treated XLT-15. In a transient expression assay, the up-regulation of MMP-9TH mRNA is dependent on thyroid hormone response elements (TREs), relative positions of which are conserved between MMP-9TH promoters of *Xenopus laevis* and *tropicalis*.

Materials and Methods

Animals

Xenopus laevis tadpoles were obtained from local breeder and were kept in dechlorinated water. *Xenopus tropicalis* tadpoles (Nigerian line) were the generous gifts from the National Bioresource Project (Institute for Amphibian Biology, Hiroshima University). Tadpoles were staged according to Nieuwkoop and Faber (Nieuwkoop & Faber 1956). All operations were performed after animals were anesthetized with 0.02% MS-222 (Sigma).

Cloning of Xenopus laevis MMP-9TH cDNA

Five hundred of cDNA clones in pBluescript (pBS) were picked up randomly from a cDNA library of stage 62 *Xenopus laevis* tadpole tail. Each insert was amplified by polymerase chain reaction (PCR) using vector primers, 5'-AATTAACCCTCACTAAAG-3' (T3 primer) and 5'-TAATACGACTCACTATAG-3' (T7 primer), electrophoresed in agarose gel, and blotted onto Nytran filters (Schleicher & Schuell). The blots were hybridized with ^{32}P-labeled cDNAs prepared from poly-(A)+ RNAs extracted from XLT-15 cells cultured in the absence and the presence of 10 nM T_3 successively. Only one clone was obtained which was expressed in stage 62 tail and T_3-treated XLT-15, but not in stage 57 tail and unstimulated XLT-15. By using this insert, a cDNA library of stage 62 tadpole tail was screened, and one positive clone contained 280 bp upstream region. Based on its sequence, the 5' rapid amplification of cDNA ends (5' RACE) was undertaken using 5'-GACCCTGTGAAATATAGT-3' as a 5RT primer and 5'-CATCATCACTGCAGCTGA-3' as a 5'amp primer (Frohman *et al.* 1988), and 1.3 kb cDNA fragment was cloned which includes an open reading region more than 300 bp. A cDNA library was constructed from poly-(A)+ RNA of stage 62 tadpole tail using 5'-AGTAATGGTCACTGCCGT-3' as a primer which is located downstream of the open reading region. After screening by the 1.3 kb cDNA fragment cloned by 5' RACE, two clones with about 2 kb insert were isolated, and sequenced. A clone with a longer insert was characterized furthermore, and designated as Xl MMP-9TH.

Northern Blot Analysis

Total RNA was extracted from cultured cells and tadpole tissues by using guanidinium thiocyanate (Chirgwin *et al.* 1979), purified by cesium chloride centrifugation, electrophoresed, blotted to a Nytran filter, and hybridized as described (Yaoita & Brown 1990). The membrane was stained with methylene blue to check the quantity and quality of the RNA. To further standardize the amounts of RNA, the *Xenopus laevis* elongation factor 1-α (EF) cDNA (a gift of Dr. Krieg) (Krieg *et al.* 1989) was also used as a probe.

In situ *Hybridization*

The Xl MMP-9TH cDNA clone in pBS was linearized with *Xba*I and *Xho*I for the preparation of antisense and sense RNA probes, respectively. The probes were synthesized and labeled with the digoxigenin-UTP using DIG RNA labeling kit (Roche Molecular Biochemicals) by T3 (an antisense probe) and T7 RNA polymerase (a sense probe) according to the manufacture's instruction.

Tadpoles were perfusion-fixed with 4% paraformaldehyde in APBS (100 mM NaCl, 10 mM phosphate buffer) and preserved in same solution overnight at 4˚C. They were infiltrated overnight in APBS containing 15% sucrose and frozen in Tissue-Tek O. C. T. Compound (Sakura) on dry ice. Sections were cut at 20 μm on a cryostat at -20˚C, placed on APS (3-Aminopropyltriethoxysilane)-coated slides and dried. *In situ* hybridization was performed as described previously (Uetsuki *et al.* 1996) using sense or antisense probe of Xl MMP-9TH. Immersion of the sections in a proteinase K solution was omitted.

Real-Time Reverse Transcription PCR (RT-PCR)

The quantitative real-time RT-PCR was carried out using a LightCycler (Roche Molecular Biochemicals) according to the instructions provided by the manufacture. Reverse transcription was performed using 0.5 μg of total RNA and PowerScript™ (Clontech) with Xl EF specific primer 5'-CTCAGTAAGGGCTTCATGG-3' and Xl MMP-9 primer 5'-GACATCACTCCAGACTTTGA-3' which was designed to a common sequence between both Xl MMP-9TH and Xl MMP-9 genes. Real-time PCR was carried out using the SYBR® Premix ExTaq™ (TaKaRa). Primers for Xl MMP-9 were 5'-CGCTCACCATTATGCAACAG-3' and 5'-GTTCAGAATACGGTAAGTGAT-3'. Primers for Xl MMP-9TH were 5'-CTCTCAGCCAAATGCAAAGT-3' and 5'-GTTTAGGATACGATATGTGAG-3'. As a control, the expression of Xl EF gene was detected using primers 5'-AAGAAGGATCTGGCAGCGG-3' and 5'-TTTAATGACACCAGTTTCCACA-3'. For *Xenopus tropicalis*, the following primers were used: Xt EF specific primer 5'-CTCGCTAAGGGCTTCATGG-3' and a common Xt MMP-9 primer 5'-CATCGAAGGGGTAAGGATC-3' for reverse transcription, 5'-AAGAGCAAGGCTCAAATGTCA-3' and 5'-GTGTTTCGGTATCTAGGACT-3' for Xt MMP-9,

5'-TCCAGCAGGGCCCATCCAA-3' and 5'-ACGTTTCTGCATCCAGGTTG-3' for Xt MMP-9TH, and 5'-AAGAGGGATCTGGCAGCGG-3' and 5'-AAGGACACCAGTCTCCACAC-3' for Xt EF. The level of specific mRNA was quantified at the point where the LightCycler System detected the upstroke of the exponential phase of PCR accumulation, and normalized to the level of the *Xenopus* EF mRNA in each sample. No RT control was also run and gave negative results.

Gelatin Zymography

MMPs were extracted from tadpole tissues and cultured cells (Talhouk *et al.* 1991). Protein in extracts was quantified using BCA Protein Assay Reagent (PIERCE). Samples were electrophoretically separated under nonreducing conditions in 10% polyacrylamide gels impregnated with 3 mg/ml gelatin. After electrophoresis, the gels were treated as previously described (Heussen & Dowdle 1980).

Preparation and Purification of Anti-Xl MMP-9TH Antibody

The peptide corresponding to the amino acid sequence of Xl MMP-9TH (residues 162-175, SGEPDINIMFGTEN) was synthesized and coupled to keyhole limpet hemocyanin as a carrier using glutaraldehyde. An antiserum against Xl MMP-9TH was prepared by injecting rabbits intradermally with the conjugate of the synthetic peptide and carrier. For affinity purification, 10 mg of the oligopeptide was bound to Affi-Gel 15 (1 ml bed volume, Bio-Rad) in 100 mM 3-[N-Morpholino]propanesulfonic acid, pH 7.5, according to the manufacture's directions. Rabbit sera were partially purified by ammonium sulfate precipitation method, and repeatedly recirculated through the oligopeptide-conjugated Affi-Gel 15. After washing with phosphate buffered saline (PBS), the specific antibody was eluted with 0.2 M glycine, pH 2.8. The fractions were immediately neutralized with saturated K_2HPO_4 and dialyzed against PBS.

Immunoblot Analysis

Samples were resolved by SDS-polyacrylamide gels under the reducing conditions, and electrotransferred onto Immobilon membranes (Millipore, MA) in a semidry blotter. After blocking nonspecific binding of antibody by bovine serum albumin, the blot was reacted with the antiserum against Xl MMP-9TH, followed by incubation with a horseradish-peroxidase-labeled goat anti-rabbit IgG antibody. Immunoblots were visualized by Western blot chemiluminescence reagent (DuPont, MA) according to the manufacturer's instructions.

Isolation of genomic clone

A λ genomic library of *Xenopus laevis* (Stratagene, CA) was screened with the Xl MMP-9TH cDNA fragment. A positive clone was isolated after the secondary screening. Restriction maps of the genomic clone were determined by digestion with various restriction enzymes. The transcription start site was determined by 5' RACE (Frohman *et al.* 1988). Numbering of Xl MMP-9TH gene begins at the start site of transcription as +1. A 4.9 kb *Eco*RI-*Hin*dIII fragment (-3092 to +1819) was isolated from the genomic clone and ligated into *Eco*RI-*Hin*dIII-digested pBS to use as a 'wild' fragment (pBS-3092/+1819 Xl MMP-9TH). This subclone was sequenced using multiple specific primers on both strands to generate overlapping sequences.

Construction of Luciferase Vectors

Regions of Xl MMP-9TH promoter and the first intron were ligated into the pEGFP-1 vector (Clontech), and then recloned in pGL3-Basic vector (Promega).

First, DNA fragment from -964 to +1299 of Xl MMP-9TH was amplified by PCR using 5'-GGGGTACCGTATTGGGCACCATTAAA-3' and 5'-GGGGATCCCTGCTGGAGGGGCA-3'. PCR product was inserted into *Sma*I site of pBS through blunt-end ligation to generate pBS-964/+1299 Xl MMP-9TH.

Site-directed mutagenesis construct was created by using PCR based approach as described (Imai *et al.* 1991), where oligonucleotides primers were designed in inverted tail-to-tail directions to amplify the cloning vector together with target sequence. After the PCR with these primers, amplified

linear DNA was self-ligated. Mutation and deletion in the Xl MMP-9TH gene were confirmed by sequence analysis. Oligonucleotides were generated to sequentially disrupt two ATG of the first exon in pBS-964/+1299 Xl MMP-9TH and to direct the translation initiation from a reporter gene (pBS-964/+1299 Xl MMP-9THM). Each ATG was mutated to GTG using 5'-TGGGTGGGCTGTGTGTTT-3' and 5'-CAATGACACCTAGGAATGC-3' for the first ATG mutation, and 5'-TGACTAGTGTGGAGGTGG-3' and 5'-CGTTTTTACGGATTTCCCCT-3' for the second ATG mutation. Accidentally, we obtained pBS-964/+1299 Xl MMP-9THMΔint which has the mutation of the first ATG and the deletion of a portion of the first exon including the second ATG and the first intron (nucleotides +82/+1251) by PCR error in addition to the desired mutant. pBS-964/+1299 Xl MMP-9THM and pBS-964/+1299 Xl MMP-9THMΔint were digested with *Bam*HI, and then the digested fragments were ligated into pEGFP-1 vector at *Bam*HI site to create -964/+1299 Xl MMP-9THM-GFP and -964/+1299 Xl MMP-9THM-GFPΔint, respectively. The -3092/+1299 Xl MMP-9THM-GFP was constructed by inserting the distal promoter (-3092/-292) prepared from *Hin*dIII-*Xba*I double digests of pBS-3092/+1819 Xl MMP-9TH into *Hin*dIII-*Xba*I sites of -964/+1299 Xl MMP-9THM-GFP. DNA from -4194 to -251 of Xl MMP-9TH was amplified by PCR with the PrimeSTAR^{TM} polymerase (TaKaRa) using a λ genomic clone DNA of Xl MMP-9TH, 5'-CCGAGCTCTATGCTTGTGTTGCTCC-3' (xMMP9-Sac) and 5'-CAGTCACATAATGACAATCC-3'. PCR products were digested with *Xba*I, and ligated to -964/+1299 Xl MMP-9THM-pEGFP1 digested with *Xba*I-*Not*I. Ligated product was amplified by PCR using xMMP9-Sac and 5'-CCTCGCCCTTGCTCACCA-3'. PCR products were digested with *Sac*I and *Bam*HI, and ligated into pGL3-Basic vector at *Sac*I-*Bgl*II site to generate -4194/+1299 Xl MMP-9THM-Luc.

Serial deletions from the 5' ends of -3092/+1299 Xl MMP-9THM-GFP and -964/+1299 Xl MMP-9THM-GFP were also created by PCR based method (Imai *et al.* 1991) using oligonucleotides primers designed in inverted tail-to-tail directions to amplify the cloning vector together with target sequence. A reverse primer 5'-CTCGAGATCTGAGTCCGGTAG-3' was generated beginning at the pEGFP-1 *Xho*I site. A series of forward primers are as follows: xMMP9ProDel-1957 5'-AGGCTGAATAGTTGCCGACG-3', xMMP9ProDel-475 5'-TCCCAGACCTGTAGTAGT, and

xMMP9ProDel-152 5'-TGATACTCACGGCTCCTTGT-3'. A series of 5' promoter deletions are indicated by the numbers of beginning and ending nucleotides.

The -964/+1299 Xl MMP-9THM-GFP and -964/+1299 Xl MMP-9THM-GFPΔint were digested with *Bam*HI, and then the digested fragments were ligated into pGL3-Basic vector at *Bgl*II site to create -964/+1299 Xl MMP-9THM-Luc and -964/+1299 Xl MMP-9THM-LucΔint, respectively. Serial deletion fragments were cut out from plasmids, ligated into pBS at *Xho*I and *Bam*HI sites, and then inserted into pGL3-basic vector at *Kpn*I and *Bgl*II sites. The -3092/+1299 Xl MMP-9THM-GFP was recloned into pGL3-Basic vector at *Sac*I and *Hin*dIII sites.

The TRE1 (+745/+760) and TRE2 (+771/+786) in the first intron were deleted from -964/+1299 Xl MMP-9THM-Luc using 5'-TGAGTATATTTGTCCTTGTC-3' and 5'-CAGAGACATAGTATAAGGCA-3' (Xl TRE1del-3’) to generate -964/+1299 Xl MMP-9THM-LucΔTRE1, and 5'-TGTAAATCTTGACTTTCTCTTACTC-3' and 5'-AATATACTCAAGGTTACAGCAGGT-3' to generate -964/+1299 Xl MMP-9THM-LucΔTRE2. A construct devoid of both TRE1 and TRE2 (-964/+1299 Xl MMP-9THM-LucΔTRE1&2) was produced from TRE2-deleted Luc vector using 5'-TGAGTATATTTGTAAATCTTGACT-3' and Xl TRE1del-3’.

Transfection and luciferase assays

XLT-15 cells were maintained at 20˚C as described previously (Yaoita & Nakajima 1997). XLT-15 cells in Multiwell^{TM} Primaria^{TM} 24-well tissue culture plates (Becton Dickinson) were transfected with 25 ng Xl MMP-9THM-Luc, 12.5 ng of *Xenopus* thyroid hormone receptor (TR) α expression vector, 12.5 ng *Xenopus* retinoid X receptor (RXR) α expression vector, and 5 ng of pRL-CMV (Promega) as an internal control using FuGENE6 transfection reagent (Roche) according to the instructions of the manufacturer. Two days after transfection, cells were incubated for 24 hours in the presence or absence of 10 nM T_3 at 25˚C. Cells were lysed, and luciferase activities were determined for firefly luciferase and *Renilla* luciferase using the Dual-Luciferase Assay System (Promega). Luciferase activities were measured in a TD 20/20 Luminometer (Turner Designs).

Gel mobility shift assay

Xenopus TRα and RXRα proteins were synthesized *in vitro* using the TNT kit (Promega) according to the manufacture's instruction. The sequences for the MMP-9TH TRE binding experiments of one strand of oligonucleotides are as follows: 5'-CCTAGGC**AGGTCA**TTTC**AGGACA**GCCCAGC-3' for *Xenopus* TRβ TRE, 5'-GTCTCTG**TTACCT**GCTG**TAACCT**TGAGTAT-3' for Xl MMP-9TH TRE1, 5'-GTCTCTG**TTATTT**GCTG**TAACCT**TGAGTAT-3' for Xl MMP-9TH mTRE1 (the nucleotides changed from wild type are underlined), 5'-GTATATT**TGTCCT**TGTC**TTACCT**TGTAAAT-3' for Xl MMP-9TH TRE2, 5'-GTATATT**TGTTTT**TGTC**TTACCT**TGTAAAT-3' for Xl MMP-9TH mTRE2, 5'-CCCATTC**TGTCCT**TAGT**TTACCT**GCTTTGC-3' for Xl MMP-9TH TRE3, and 5'-CCCATTC**TGTTTT**TAGT**TTACCT**GCTTTGC-3' for Xl MMP-9TH mTRE3. Bold letters indicate the TRE half-sites. Oligonucleotides were annealed by heating in 20 mM Tris-HCl (pH 7.5) 10 mM $MgCl_2$, and 50 mM NaCl to 95°C for 5 min and then cooled to room temperature for use as probes. The probes were labeled using T4 polynucleotide kinase and $[\gamma\text{-}^{32}P]$ATP. After stopping the reaction, unicorporated nucleotides were removed by Sephadex G-50 columns (Amersham). Two μl each *in vitro* translated TRα and RXRα and 1 μl of the end-labeled TRE fragment were added together with unlabeled competitor, and the mixture was incubated at room temperature for 20 min in 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, and 100 ng of poly(dI·dC) in a final volume 20 μl. Samples were electrophoresed on 6% nondenaturing polyacrylamide gels in 0.5× TBE buffer at 150 V for 90 min. Gels were dried and analyzed using a bio-image analyzer (Fuji BAS-2000, Fuji photofilm).

Results

Isolation of a novel Xenopus laevis MMP-9 gene, Xl MMP-9TH

XLT-15 is a myoblastic cell line derived from *Xenopus laevis* tadpole tail and undergoes apoptosis in the presence of TH (Yaoita & Nakajima 1997). A regressing tail cDNA library was screened to isolate

a gene which is expressed specifically in stage 62-63 regressing tails and T_3-stimulated XLT-15 myoblastic cells. One of the isolated clones was sequenced and found to encode a protein with a similarity to all known MMP-9s, and to have the strongest homology to previously described *Xenopus laevis* MMP-9 (Carinato *et al.* 2000; Yang *et al.* 1997). In order to distinguish these MMP-9 genes, a newly cloned gene was designated as Xl MMP-9TH, because this gene expression was induced by TH as described below. *Xenopus laevis* genome is tetraploid at most loci (Bisbee *et al.* 1977; Graf & Kobel 1991), and two pseudoalleles can be found for many genes. *Xenopus laevis* pseudoalleles share over 95% amino acid identity in the cording region (Bergwitz *et al.* 1998; Yaoita *et al.* 1990). However, deduced amino acid sequences of Xl MMP-9TH and Xl MMP-9 are only 78% identical (Fig. 1A). Alignment of the deduced Xl MMP-9 TH protein sequence with the previously reported *Xenopus laevis* and *tropicalis* MMP-9s is presented in Fig. 1B. Furthermore, we found that the diploid anuran *Xenopus tropicalis* have both MMP-9TH and MMP-9 genes by searching in the *Xenopus tropicalis* database (DOE Joint Genome Institute), although there is a single MMP-9 gene in human, mouse, rat, chicken, and zebrafish genomes. MMP-9TH gene is located 9 kb downstream of MMP-9 gene on the chromosome in the same transcriptional orientation in the *Xenopus tropicalis* genome (Fig. 1C). These observations strongly suggest that Xl MMP-9TH and Xl MMP-9 represent two distinct genes, but not pseudoalleles due to the tetraploidy of *Xenopus laevis* genome.

The domain structure is conserved among these MMP-9s, and can be separated into five distinct functional domains: the pre-peptide, the pro-domain, the catalytic domain including fibronectin type II repeats, hinge region and hemopexin/vitronectin domain from the N terminus. The cysteine switch (PRCGXPD) is a most conserved sequence in the pro-domain of MMP family and necessary to mask the active site of enzyme. Glycine at the forth residue of the cysteine switch was replaced with alanine in Xl MMP-9TH gene. This variation was confirmed by the sequences of another independent cDNA clone, genomic DNA fragment and RT-PCR product which was prepared by the reverse-transcriptase reaction using crude RNA of stage 62 tadpole tails (data not shown). Xl MMP-9TH gene encodes a functional gelatinase, because the conditioned medium of Xl MMP-9TH-transfected cultured cells contained a gelatinolytic activity on zymogram (Fig. 5C, lane 4).

The expression of Xl MMP-9TH mRNA was induced in degenerative tissues during metamorphosis and T_3-treated XLT-15

Northern blot analysis was performed to examine the expression of Xl MMP-9TH mRNA during the spontaneous metamorphosis using total RNAs extracted from tail, hind limb, intestine and central nervous system (Fig. 2A). In tail, MMP-9TH mRNA started rising at stage 61 following the increase of endogenous TH from stage 58, and was up-regulated finally more than 1000-fold at stage 63 compared with stage 58. The notochord in tail begins to degenerate at stage 61, and the degeneration of the muscle segments proceeds very rapidly from stage 62 (Nakajima & Yaoita 2003; Nieuwkoop & Faber 1956). On the other hand, a level of MMP-9TH transcript was very low and did not change significantly in growing hind limb during the same period. The expression of MMP-9TH gene in intestine and the central nervous system reached a peak at stage 60-61 and 62, respectively. In intestine, apoptosis of larval epithelial cells is observed frequently during stage 59-61 (Ishizuya Oka & Shimozawa 1992) and it is known that cell death frequency peaks at stage 62 in mesencephalic fifth nucleus (Kollros & Thiesse 1985), although no conspicuous change takes place in the central nervous system except topographical changes during the climax of metamorphosis (Nieuwkoop & Faber 1956). These results show a correlation of the MMP-9TH expression with cell death and tissue degeneration during metamorphosis, although the maximal expression was observed at different stages in different organs.

Since the expression profile of Xl MMP-9TH was most correlated with cell death and tissue degeneration, Xl MMP-9TH expression was examined in details by *in situ* hybridization analysis using gills that disappear in metamorphosing tadpole. The degeneration of the branchial arches begins at stage 60 and is completed by stage 63. *In situ* hybridization analysis revealed that branchial arches expressed a large amount of the mRNA at stage 61, but not at stage 57 before the climax of metamorphosis (Fig. 3), suggesting a tight correlation of Xl MMP-9TH expression with tissue degeneration.

To confirm the T_3-induction of Xl MMP-9TH mRNA in a myoblastic cell line derived from stage 57 tadpole tail, Northern blot analysis was performed using RNAs from XLT-15 cells stimulated with 0 to 10 nM T_3 (Fig. 2B). XLT-15 cells could respond to 0.3 nM T_3 in 24 hours, and the amount of Xl MMP-9TH mRNA increased at the higher concentration of T_3. The expression of Xl MMP-9TH mRNA started within two hours after T_3-treatment of XLT-15 cultured cells, and increased gradually as seen in TH-induced expression of TH/bZIP gene (Furlow & Brown 1999) (Fig. 2C). The expression of both TH/bZIP and Xl MMP-9TH genes has an early low level of up-regulation, followed several hours later by the greatest part of the response. A report that TH/bZIP gene expression is regulated *in vitro* through TREs implies a possibility that TH-induced expression of Xl MMP-9TH is also mediated by TRE. This idea is also supported by the observation that the up-regulation of Xl MMP-9TH by TH was partially resistant to the inhibition of protein synthesis as that of TH/bZIP (Wang & Brown 1993) (Fig. 2D).

Since the coding region of Xl MMP-9TH has 82% identify to Xl MMP-9 at the nucleotide level, we cannot exclude a possibility that Xl MMP-9TH probe cross-hybridized to Xl MMP-9 mRNA. To precisely determine expression levels of Xl MMP-9 and Xl MMP-9TH mRNAs, we used quantitative RT-PCR with gene-specific primers. The specificity of these primers was confirmed using plasmid DNA containing Xl MMP-9 or Xl MMP-9TH gene. MMP-9TH mRNA was strongly induced more than 1800-fold in stage 63 tail of *Xenopus laevis* and *tropicalis* and 25-fold in stage 62 central nervous system of *Xenopus laevis*, but not MMP-9 mRNA (Fig. 4A,B). In intestine, Xl MMP-9TH and Xl MMP-9 mRNAs peaked at stage 60 and 61, respectively (Fig. 4C). Apoptosis of larval epithelial cells is most frequently observed at stage 59-61 (Ishizuya Oka & Shimozawa 1992), whereas nests of adult epithelial cells appear at stage 60, increase size, and replace larval epithelium almost completely by stage 62 (McAvoy & Dixon 1977). It appears that Xl MMP-9TH mRNA is expressed at the maximal level when larval epithelial cells die, and that the expression of Xl MMP-9 mRNA reaches a peak when adult epithelial cells proliferate. In a myoblastic cell line, XLT-15, T_3-treatment induced exclusively MMP-9TH mRNA 280-fold compared with untreated XLT-15 (Fig. 4D). However, in embryos (stage 39) MMP-9 mRNA was expressed fifty times more than MMP-9TH

mRNA (Fig. 4E). These results show that Xl MMP-9TH gene was up-regulated by TH, and that its expression is correlated with cell death and degeneration of organs during metamorphosis.

A gelatinolytic activity of Xl MMP-9TH was strongly induced in regressing tail and T_3-treated XLT-15

Since MMP-9 gene encodes a gelatinase, we performed gelatin zymography to examine a gelatinolytic activity in regressing tail during the spontaneous metamorphosis and in T_3-treated XLT-15 cultured cell line. A strong gelatinase activity was detected only in regressing tail, but not in tail before stage 61 or in hind limb (Fig. 5A). The similar result was also observed in regressing tail of *Xenopus tropicalis* tadpole. A gelatinolytic activity was prominently induced in conditioned medium of T_3-treated XLT-15 (Fig. 5B), but not in that of T_3-treated adult kidney fibroblastic cell line A6 (data not shown).

To test whether cloned Xl MMP-9TH gene encodes a functional gelatinase, XLT-15-11 cells (Nakajima *et al.* 2000) was transfected with Xl MMP-9TH expression construct. Its conditioned medium contained a potent gelatinolytic activity on zymogram (Fig. 5C). Both conditioned media of Xl MMP-9TH-transfected cells and T_3-treated myoblastic cell line showed a gelatinase activity with the same molecular weight.

To examine whether the detected gelatinase is derived from Xl MMP-9TH, we compared a gelatin zymogram and immunoblot using the antiserum raised against Xl MMP-9TH (residues 162-175, SGEPDINIMFGTEN). The antiserum recognized molecules that correspond to gelatinase species on zymogram in T_3-treated XLT-15 and a regressing tail (Fig. 5D). MMP is secreted in zymogen form except membrane type MMPs and stromelysin, and activated by proteolytic processing (Woessner 1991). T_3-treated XLT-15 cells (Fig. 5C, lane 2) and Xl MMP-9TH-transfected cells (Fig. 5C, lane 4) secret 92-kDa gelatinase that is a proenzyme, while an active form of 73-kDa gelatinase was detected in the extracts of regressing tail of stage 63 tadpole (Fig. 5D, lane 8) and TH-treated tails (data not shown). We concluded that this 73-kDa gelatinase is MMP-9TH, but not MMP-2, because anti-MMP-9TH serum recognized 73-kDa gelatinase, and there is no change in the overall amount of MMP-2 species in organ-cultured tails treated with T_3 on gelatin zymogram in spite of the up-regulation of

MMP-2 mRNA (Jung *et al.* 2002). In addition, the molecular weight sizes of proMMP-2 and its activated form are 72 and 62 kDa in human cultured cells, respectively (Ramos-DeSimone *et al.* 1999). As the gelatinase in regressing tail should be the active form, it does not appear to be MMP-2 protein. We believe that anti-MMP-9TH serum did not detect endogenous MMP-9 protein in Fig. 5D, because XLT-15 cells and tadpole tails expressed hardly any MMP-9 mRNA (Fig. 4A,D), and the amino acid sequence is different in a region used for the immunization between MMP-9TH and MMP-9 proteins (Fig. 1B). Zymograms were blank when gels were developed in the presence of 10 mM EDTA or 0.5 mM 1, 10-phenanthroline (data not shown), consistent with the identity of all gelatinase species as MMPs.

These results are compatible with the expression profile of Xl MMP-9TH mRNA and show the correlation of tissue resorption by TH with the induction of a gelatinase activity due to MMP-9TH.

Genomic organization of Xl MMP-9TH gene

Towards a better understanding of transcriptional regulation of the Xl MMP-9TH gene by TH, we have determined its genomic organization and promoter structure. Xl MMP-9TH genomic clone was isolated by screening λ genomic library using a Xl MMP-9TH cDNA probe. The genomic structure of the Xl MMP-9TH gene is illustrated in Fig. 1C. The initiation site for transcription was determined by the 5’ rapid amplification of cDNA ends (5'-RACE). A TATA-like box was found at -33 relative to the transcription start site. We obtained *Xenopus tropicalis* MMP-9TH and MMP-9 genomic sequences from JGI genome database. The alignment of MMP-9TH promoter sequences of *Xenopus laevis* and *tropicalis* showed that the relative positions of a TATA-like box, two AP-1 binding sites, and an AML-1 binding site (Meyers *et al.* 1993) are conserved. The *Xenopus laevis* and *tropicalis* MMP-9TH sequences share 86% identity for 300 bp upstream of the translational start site, whereas *Xenopus tropicalis* MMP-9 has 63-64% homology for 200 bp upstream of the translation start site with *Xenopus laevis* or *tropicalis* MMP-9TH promoter sequences.

We searched for putative TRE in *Xenopus laevis* and *tropicalis* MMP-9TH genes and *Xenopus tropicalis* MMP-9 gene by using Internet site NHR Scan, available at

http://mordor.cgb.ki.se/NHR-scan (Sandelin & Wasserman 2005). Two putative binding sites for TR are located in the first intron, and one is found approximately 3.5 kb upstream of the initiation codon in *Xenopus laevis* and *tropicalis* MMP-9TH genes (Fig. 1C). These TRE-like sequences consist of two near perfect repeats of AGGTCA separated by 4 bp (Table 1). Another TRE-like sequence is present in the 9th intron in Xt MMP-9TH gene, but not conserved in Xl MMP-9TH gene (data not shown). In MMP-9 gene of *Xenopus tropicalis*, two TRE-like sequences are located 6.3 and 10 kb upstream of the initiation codon.

The NF-κB site, which is highly conserved among MMP-9 promoters of the mammalian species, was not identified in the promoter sequences of *Xenopus laevis* and *tropicalis* MMP-9TH and of *Xenopus tropicalis* MMP-9, and the microsatellite segment of alternating CA residues that is found in the rat, human, and mouse MMP-9 promoters was also absent in the *Xenopus* sequences (Campbell *et al.* 2001; Munaut *et al.* 1999).

Functional analysis of the Xl MMP-9TH gene promoter

Our initial experiments were designed to define regions of the Xl MMP-9TH gene that were involved in the stimulation of expression by T_3. To conduct these experiments, the MMP-9TH promoter sequence was ligated in front of the luciferase reporter gene in the pGL3-Basic vector. We performed transient expression assays using luciferase reporter gene driven by various lengths of the Xl MMP-9TH 5'-flanking region. These assays were examined by using XLT-15 cells, because these cells were induced by T_3 treatment to express and secrete Xl MMP-9TH, as described above (Fig. 2B and 5B). The -3092/+1299 Xl MMP-9TH-Luc vector contains the region from -3092 to +1299 relative to the transcription start site upstream of the luciferase gene. Mutations (ATG to GTG) were introduced in the ATG initiation codons in the first exon in order to allow translation of the transcript to start from the first ATG initiation codon in the luciferase gene (-3092/+1299 Xl MMP-9THM-Luc).

Addition of T_3 stimulated the transcription of the -3092/+1299 Xl MMP-9THM-Luc vector 10-fold (Fig. 6C). The better T_3-induction was observed by transfection with -4194/+1299 Xl MMP-9THM-Luc vector that contains the additional TRE3 (nucleotides -3311 to -3296). This raises the

possibility that TRE-like sequence that is located 3.3 kb upstream of the transcription initiation site is functional (Fig. 6B). Sequential deletion from -3092 to -964 resulted in the gradual decrease of T_3-induction, which implies the presence of some positive regulatory regions other than TRE. This is supported by the existence of the conserved regions between *Xenopus laevis* and *tropicalis*, nucleotides -3092 to -2809 and nucleotides -1496 to -1413 (Fig. 6A). These data and a partial resistance of T_3-induced activation to cycloheximide suggest a significant role of indirect regulation by TH. It is also possible that tail cells become competent to produce MMP-9TH as a response to TH during the climax of metamorphosis by expressing positive regulatory transcriptional factors that bind to such conserved regions.

Although T_3 treatment stimulated the transcription of the -964/+1299 Xl MMP-9THM-Luc vector, this induction by T_3 was completely lost in a transfection assay using the same construct in which the first intron was deleted (-964/+1299 Xl MMP9THM-LucΔint) (Fig. 6C). This is indicative of the presence of thyroid hormone responsive region in the first intron, which is consistent with the presence of two TRE-like sequences in the first intron.

To test whether TRE-like sequences in the first intron of Xl MMP-9TH gene is responsible for T_3 induction, additional deletions were generated (Fig. 7). The proper splicing of the primary transcript originating from the vectors (-964/+1299 Xl MMP-9THM-Luc and -964/+1299 Xl MMP-9THM-LucΔTRE1&2) was confirmed by RT-PCR (data not shown). A 3-fold induction by T_3 treatment was detected using -964/+1299 Xl MMP-9THM-Luc vector. T_3-induction was slightly decreased when one of TRE-like sequences in the first intron was deleted, and was completely lost upon deletion of both TRE-like sequences compared with pGL3. This shows that two putative TREs in the first intron of MMP-9TH gene are important to T_3-induction of -964/+1299 Xl MMP-9THM-Luc vector. Although it is unknown whether MMP-9 expression is up-regulated by TH, we cannot exclude a possibility that MMP-9 is induced in intestine during the climax of metamorphosis (Fig. 4C) through TRE-like sequences in MMP-9TH and MMP-9 promoter regions, since two genes are located in tandem.

Direct binding of TR to the Xenopus MMP-9 TREs

We tested whether MMP-9 TREs can form specific complexes with TR and RXR, since the relative positions of TRE-like sequences to the translational initiation site is conserved between *Xenopus laevis* and *tropicalis*, and the deletion of TRE-like sequences resulted in the loss of T_3-induction of MMP-9TH promoter. Gel mobility shift assay was performed using *in vitro* translated TR and RXR. TRE1 and TRE2 are present in the first intron of *Xenopus laevis* MMP-9TH gene, and TRE3 is located 3.5 kb upstream of the transcription initiation site. The end-labeled TRE1 and TRE2 failed to form any specific complexes with the TR or RXR alone under the detection conditions, and formed a complex with the mixture of the two proteins as TRβ TRE (Fig. 8A-C). The binding of TR/RXR complexes to labeled TRE1 and TRE2 was competed completely by a 50-fold excess of cold TRE1 and TRE2, respectively, but not by the same amounts of mutated versions of TREs. Although TRE3 gave rise to a non-specific complex with unprogrammed reticulocyte lysate, it also formed a complex with a mixture of receptor proteins that disappeared in the presence of an excess of cold TRE3 (Fig. 8D). A faster migrating complex with a control reticulocyte lysate was competed by a mutated version of TRE3 with the replacement of the G residues at the second and third positions of the second half site of TRE, whereas a slower migrating complex remained even after an addition of a mutated TRE3. The results suggest that a slower migrating complex is formed specifically with TR and RXR, and that TRE3-binding protein in unprogrammed reticulocyte lysate is not endogenous TR, because the G residues at the second and third positions of the second half site are absolutely required for binding of TR/RXR to TRE (Kurokawa *et al.* 1993; Furlow & Brown 1999).

To assess the relative binding affinities of various TRE sequences, gel mobility shift assay was performed with a radio-labeled TRβ TRE and various unlabeled competitors (Fig. 8E,F). The binding of TR/RXR to TRß TRE was competed completely with a 50-fold excess of cold TRß TRE, and efficiently with a 200-fold excess of MMP-9TH TRE1, TRE2, or TRE3, supporting that TR/RXR binds to MMP-9TH TREs in a sequence-specific manner.

Discussion

We show here that MMP-9 gene is duplicated in *Xenopus* genomes, and that expression of MMP-9TH is especially induced at a high level in regressing organs including tail and gills during the climax of spontaneous metamorphosis, whereas originally identified MMP-9 gene is expressed in embryo.

Gene Duplication of MMP-9

The genomes of extant vertebrates have been shaped by a series of whole genome and individual gene duplication events (Ohno 1970). Our study here has shown that MMP-9 gene is duplicated in *Xenopus* genomes in contrast to the presence of only one MMP-9 gene in genomes of human, mouse, rat, chicken and zebrafish. Recent data derived from analysis of young duplicate gene pairs in human genome show that one copy generally evolves faster and accumulates amino acid substitutions evenly across the sequence, whereas functional constraints act to slow the rate of mutation of the other (Zhang *et al.* 2003). However, human MMP-9 is 55-58% identical to MMP-9s and MMP-9THs of *Xenopus laevis* and *tropicalis* at the amino acid sequence level, and Japanese flounder MMP-9 also has 59-61% identity to them (Fig 1A). This suggests that the coding regions of duplicated *Xenopus* MMP-9 genes have evolved at the amino acid level at similar rates from an MMP-9 ancestor gene.

MMP-9TH has 78% homology with MMP-9 at the amino acid sequence level in both *Xenopus laevis* and *tropicalis*, while *Xenopus laevis* MMP-9TH and MMP-9 is about 90% identical to *Xenopus tropicalis* MMP-9TH and MMP-9, respectively (Fig 1A). Therefore, it is plausible that a single MMP-9 gene was duplicated, and that these genes differentiated to MMP-9 and MMP-9TH genes before the ancestor of *Xenopus laevis* diverged from that of *Xenopus tropicalis* 63.7 million years ago (Evans *et al.* 2004). Furthermore, MMP-9TH gene is highly expressed in regressing tail in *Xenopus laevis* and *tropicalis*, but not MMP-9 gene. The *Xenopus laevis* and *tropicalis* MMP-9TH promoters have 86% identity for 300 bp upstream of the translational start site (Fig. 6A), whereas *Xenopus tropicalis* MMP-9 is only 63-64% identical for 200 bp upstream of the translation start site compared with *Xenopus laevis* and *tropicalis* MMP-9TH promoters. The relative positions of three TREs are conserved between *Xenopus laevis* and *tropicalis* MMP-9TH genes. These results suggest

that the TH-inducible transcriptional regulatory region was also established before the segregation of *Xenopus laevis* and *tropicalis*, and transmitted with MMP-9TH structural gene to descendants.

Expression of MMP-9TH gene in regressing tail

The collagenolytic activity was detected using tadpole tail fin, intestine and gills in the 1960s (Gross & Lapiere 1962). Thirty years later, stromelysin-3 and collagenase-3 genes were cloned as genes of ECM-degrading enzymes that are expressed in regressing tail during the climax of metamorphosis and induced in TH-treated tadpole tail (Wang & Brown 1993). It is showed by *in situ* hybridization analysis that stromelysin-3 and collagenase-3 are up-regulated during the climax of metamorphosis in the tail subepidermal fibroblasts and the myotendinous junctions to which the muscle fibers are attached. Therefore, it is hypothesized that MMPs are induced and secreted into intercellular space by TH-stimulation, and degrade ECM including the myotendinous junctions, which releases muscle cells from ECM and causes their death. It is called a murder model (Berry *et al.* 1998). The blockade of TH-signaling by the overexpression of DNTR can inhibit cell-autonomous death before tail resorption, but not completely cell death in regressing tail during the spontaneous metamorphosis, indicating that muscle cells are killed by the TH-inducible extracellular factors in shortening tail (Nakajima & Yaoita 2003). This cell death in regressing tail is only partially suppressed by the overexpression of bcl-XL (Nakajima & Yaoita 2003) and bax antisense RNA (Sachs *et al.* 2004). It is conceivable that ECM degradation by TH-induced expression of MMP genes results in a lytic environment and a loss of cellular anchorage, leading to the cell death. Collagenase-4 (Stolow *et al.* 1996) is reported to be expressed in tail during the climax of metamorphosis in addition to stromelysin-3 and collagenase-3. In organ-cultured tails, TH treatment induces the expression of MMP-2 and MT3-MMP mRNAs, and is suggested to increase a gelatinolytic activity by MMP-9 on gelatin zymogram (Jung *et al.* 2002).

We show that MMP-9 gene is duplicated in *Xenopus* genomes, and that MMP-9TH of duplicated genes is exclusively expressed in regressing tail during anuran metamorphosis, and induced in T_3-treated tail-derived myoblastic cells. There is no gene duplication in *Xenopus tropicalis* genome

as to other MMPs that are known to be expressed in resorbing tail. These observations suggest that MMP-9TH is specialized to play an important role in the degeneration of tail. Mammalian MMP-9 cleaves type IV collagen that is a major component of the basal lamina and located under all epithelial cell sheets and tubes, between muscle cells, and in the notochord lamella. It is plausible that TH-induced expression of MMP-9TH contributes to the collapse of notochord and tail muscle cell death by degrading the basal laminae.

The addition of anti-stromelysin-3 antibody to the medium of intestinal organ cultures leads to an inhibition of TH-induced ECM remodeling, apoptosis of the larval epithelium, and the invasion of the adult intestinal primordia into connective tissue, that is, the intestinal metamorphic remodeling (Ishizuya-Oka *et al.* 2000). The heat-inducible expression of stromelysin-3 in transgenic tadpoles is sufficient to induce larval epithelial cell death and fibroblast activation, events that normally occur only in the presence of TH (Fu *et al.* 2005). As stromelysin-3 is also induced in regressing tail (Berry *et al.* 1998), it is possible that stromelysin-3 is involved in the activation process of MMP-9TH protein or collaborate with MMP-9TH to directly digest ECM and induce the degeneration of tail and the cell death.

It is reported that MMP-2 and MMP-14 can considerably potentiate the activation rate of procollagenase-3 (Knauper *et al.* 1996), and that collagenase-3 directly activates human proMMP-9 in a two-step cleavage mechanism (Knauper *et al.* 1997). MMP-2 and collagenase-3 might be induced by TH, activated, and cleave MMP-9TH into an active form. This idea is supported by the observation that a MMP inhibitor represses the processing of TH-induced proMMP-9 in organ-cultured tails (Jung *et al.* 2002). Since mice lacking both MMP-9 and collagenase-3 have revealed that collagenase-3 works synergistically with MMP-9 to degrade cartilage collagen and aggrecan (Stickens *et al.* 2004), MMP-9TH and collagenase-3 might dismantle notochord and the other tail structures in a coordinated manner.

T_3-regulated expression of MMP-9TH gene

The elaborate analysis of transcriptional activation during anuran metamorphosis is reported on three TH-induced genes, xTRβ (Ranjan *et al.* 1994; Wong *et al.* 1998), xBTEB (Furlow & Kanamori 2002) and TH/bZIP (Furlow & Brown 1999). Both xTRß and xBTEB genes are early TH response genes in all tadpole organs, lack a TATA box, and instead contain an initiator or initiator-like sequence near the transcription start site. In addition, xTRβ and xBTEB promoters contain a single TRE. On the other hand, TH/bZIP gene is an early/late TH response gene, and have a TATA box near the transcription start site. Its expression is only partially resistant to an inhibitor of protein synthesis, cycloheximide (Wang & Brown 1993). TH/bZIP gene contains two TREs just upstream of a TATA box. MMP-9TH gene shares some characteristics with TH/bZIP gene. The TH-induced expression of Xl MMP-9TH gene showed an early low level of up-regulation, followed several hours later by the majority of mRNA accumulation, and the partial resistance to cycloheximide. Both *Xenopus laevis* and *tropicalis* MMP-9TH genes have a TATA box, two TREs in the first intron, and one TRE approximately 3.4 kb upstream of the initiation codon. However, Xl MMP-9TH mRNA was weakly induced in tails of stage 56 tadpoles stimulated with T_3 for one day (data not shown) in contrast to the strong induction of TH/bZIP mRNA in tails of stage 54 tadpoles treated similarly (Wang & Brown 1993). TH could induce MMP-9TH mRNA at high levels in XLT-15 or tail of stage 57 tadpole (data not shown). We believe that XLT-15 cell line has maintained TH-response ability of stage 57 tail, because it was established from a primary culture of stage 57 tadpole tail. In the case of carnitine palmitoyltransferase Iα gene, the first intron is required for T_3 induction of this gene expression, but does not contain a TRE. Its TRE is located 3 kb upstream of the transcription initiation site. It is conceivable that looping of the DNA brings TR and the accessory factors bound to the first intron closer to each other and to the transcription start site, leading to T_3 induction (Jansen *et al.* 2000). Tail after stage 57 might have accessory factors that are not expressed in younger tail and participate in T_3 induction of MMP-9TH gene as essential transcriptional factors in a cooperated manner with TR-TH complex on TRE.

We show that TREs in *Xenopus* MMP-9TH promoter are functional in a transient expression assay and stimulate the transcription in the presence of TH. It does not mean that the analyzed MMP-

9TH promoter region can drive proper temporal and spatial expression of a reporter gene faithfully as an endogenous MMP-9TH gene *in vivo*. To confirm it, it is necessary to create transgenic tadpoles using the cloned MMP-9TH promoter linked to a reporter gene, although it is still worth noting that three TREs are conserved at the similar positions relative to the initiation codon in *Xenopus laevis* and *tropicalis* MMP-9TH promoters, and play a role in TH-induction in a transient expression assay.

Our study strongly suggests that MMP-9 gene was duplicated, and that these genes diverged at the similar rate and differentiated into MMP-9 and MMP-9TH genes in a common ancestor of *Xenopus laevis* and *tropicalis*. Not only the coding region, but also the promoter region including three TREs is conserved in *Xenopus laevis* and *tropicalis* MMP-9TH genes for more than 60 million years. MMP-9TH might contribute to the degeneration of tail and gills and the remodeling organs such as intestine and central nervous system at the climax of metamorphosis. Although there is no report so far on whether other anuran has both MMP-9TH and MMP-9 genes or not, our results point out a possibility that duplication of MMP-9 gene occurred in an anuran ancestor, and that one of MMP-9 gene, MMP-9TH, was utilized as a TH-inducible MMP-9 gene for the rapid degeneration of larva-specific organs such as tail and gills during metamorphosis.

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Figure legends

Fig. 1. The comparison of *Xenopus laevis* and *tropicalis* MMP-9 amino acid sequences. (A) The homology comparison of MMP-9 amino acid sequences. (B) Amino acid sequences of *Xenopus laevis* MMP-9s (Xl MMP-9 and Xl MMP-9TH) are aligned with those of *Xenopus tropicalis* MMP-9s (Xt MMP-9 and Xt MMP-9TH). *Xenopus tropicalis* MMP-9TH amino acid sequence is deduced from the genomic nucleotide sequence. Shaded amino acids indicate identity with Xl MMP-9TH sequence. The typical MMP domains are indicated in the figure (pre-peptide, pro-domain, catalytic domain, hinge region and hemopexin/vitronectin domain). Three fibronectin type II repeats are inserted in the catalytic domain. The cysteine switch sequences are boxed. The sequence of a synthetic peptide used for immunization of rabbits is underlined. (C) The genomic structures of Xt MMP-9, Xt MMP-9TH, and Xl MMP-9TH genes. Boxes represent exons. The putative TREs are indicated with arrowheads. Arrows show the direction of transcription.

Fig. 2. Expression of Xl MMP-9TH gene is induced during the spontaneous metamorphosis and in a T_3-treated XLT-15 cultured cell line. (A) The developmental expression of Xl MMP-9TH mRNA in tail, hind limb, intestine and central nervous system. A Xl MMP-9TH probe was hybridized to 5 μg of total cellular RNAs isolated from tail (a), hind limb (b), intestine (c) and central nervous system (d) of stage 56-63 tadpoles. These blotting filters contain RNA of stage 58 tail for the comparison. Arrowheads indicate the positions of 18S and 28S rRNAs. (B) The T_3 dose response of Xl MMP-9TH mRNA up-regulation. A Xl MMP-9TH probe was hybridized to 5 μg of total cellular RNAs isolated from XLT-15 cells treated with the indicated concentration of T_3 for 24 hours. (C) The time course of Xl MMP-9TH mRNA up-regulation by T_3. A Xl MMP-9TH probe was hybridized to 5 μg of total cellular RNAs isolated from XLT-15 cells treated with 10 nM T_3 for the indicated times. (D) The effect of protein synthesis inhibition on Xl MMP-9TH mRNA up-regulation by T_3. A Xl MMP-9TH probe was hybridized to 5 μg of total cellular RNAs isolated from XLT-15 cells treated with or without 10 nM T_3 for 8 hours in the presence or absence of a protein synthesis inhibitor, 10 μg/ml cycloheximide. Control hybridization of the blots with the *Xenopus laevis* elongation factor-1α probe (EF) (Krieg *et al.* 1989) is shown below to standardize the amounts of RNAs.

Fig. 3. *In situ* hybridization of Xl MMP-9TH in the branchial arches. Two consecutive cross sections of branchial arches in stage 57 (A,B) and stage 61 (C,D) tadpole were hybridized with antisense (A,C) or sense (B,D) digoxigenin-labeled Xl MMP-9TH probe.

Fig. 4. Quantitative real-time RT-PCR analysis of MMP-9 and MMP-9TH mRNA expression. Total RNA was extracted and subjected to quantitative real-time RT-PCR to determine MMP-9 and MMP-9TH mRNA levels normalized to EF expression. Real-time RT-PCR was performed using total RNAs isolated from tails of stage 57 and 63 tadpoles of *Xenopus laevis* and *tropicalis* (A), central nervous system of stage 57 and 62 tadpoles (B), intestine of stage 57-63 tadpoles (C), XLT-15 cells cultured in the absence (control) or presence of 10 nM T_3 for 20 hours (D), and whole embryo of stage 39 (E). Results are the means ± S.E. of three independent experiments. The levels of MMP-9 and MMP-9TH mRNAs are shown as copy numbers relative to 10,000 copies of EF mRNA.

Fig. 5. Detection and identification of gelatinolytic enzymes by zymographic and immunoblotting analyses. (A) Gelatin zymographic analysis of *Xenopus laevis* tail and hind limb and *Xenopus tropicalis* tail. Each lane contains 1 μg total protein at the indicated stage. (B) Gelatin zymographic analysis of conditioned media from TH-treated XLT-15 cultured cells. Each lane contains 10 μl of conditioned medium from the cultured cells treated without or with 10 nM T_3. The numbers above the lanes refer to days of T_3-treatment. (C) Gelatin zymographic analysis of conditioned media from the cultured cells transfected with Xl MMP-9TH expression construct. Each lane contains 10 μl of conditioned medium. XLT-15-11 cultured cells (Nakajima *et al.* 2000) were treated without (lane 1) or with 10 nM T_3 (lane 2) for three days. Alternatively, XLT-15-11 cultured cells were transfected with a vector (lane 3) or Xl MMP-9TH expression construct (lane 4) and incubated for three days. (D) Comparison of zymogram and immunoblot reacted with anti-Xl MMP-9TH serum. The upper and lower panels are gelatin zymogram and immunoblot using rabbit anti-Xl MMP-9TH serum, respectively. In the gels for zymographic and immunoblot analyses, 2 and 12 μl of conditioned medium of XLT-15 cells cultured for 5 days without or with 10 nM T_3 (lanes 1 and 2), 11 and 66 μg of total cellular protein from XLT-15 cells treated for 3 days in the absence or presence of T_3 (lanes 3 and 4), and 3 and 20 μg of protein from hind limb (lanes 5 and 6) or tail (lanes 7 and 8) of stage 56 and 63 tadpoles were loaded, respectively.

Fig. 6. Transient transfection assays of luciferase expression driven by Xl MMP-9TH promoter. (A) Sequence comparison of genomic DNA surrounding the promoter in Xl and Xt MMP-9TH genes. The percentage nucleotide sequence identity in every 25 bp is indicated on the diagram. The MMP-9TH gene structure is represented below. The exons are indicated by dark boxes. The numbers show distance from the transcription initiation site (+1). The locations of putative TREs in the promoters of *Xenopus laevis* and *tropicalis* are indicated with arrows labeled by l and t, respectively. (B,C) XLT-15 cells were transiently cotransfected with 25 ng of the indicated constructs, 12.5 ng of xTRα expression vector, 12.5 ng xRXRα expression vector and 5 ng of pRL-CMV. Two days after transfection, cells were incubated for 24 hours in the presence or absence of 10 nM T_3. Cells were lysed, and luciferase assays were conducted. When xTRα and xRXRα expression vectors were removed in transient transfection assays, the fold induction by T_3 decreased to 1.1 and 1.2, respectively. Each point was performed in triplicate and repeated at least three times. The data are presented as fold induction by T_3. The error bars represent the S.E.

Fig. 7. Identification of functional T_3 responsive elements in the first intron of Xl MMP-9TH gene. The regions of nucleotides +204 to +1258 (a portion of the first exon and almost all the first intron), nucleotides +745 to +760, nucleotides +771 to +786, and nucleotides +745 to +760 and nucleotides +771 to +786 were deleted to generate the -964/+1299 Xl MMP-9THM-LucΔint, ΔTRE1, ΔTRE2, and ΔTRE1&2, respectively. These vectors were transfected as described in the legend to Fig. 6. Each point was conducted in triplicate and repeated three times. Results are expressed as fold induction by T_3. The error bars represent the S.E.

Fig. 8. The binding of MMP-9TH TREs to *in vitro* translated TR and RXR. Gel mobility shift assay was carried out using the end-labeled TRβ TRE (A,E,F), MMP-9TH TRE1 (B), TRE2 (C), or TRE3 (D). A labeled TRE was incubated in the presence of unprogrammed reticulocyte lysate (URL) or *in vitro* translated TR or/and RXR with or without a 50-fold (A-E) or 200-fold (F) excess of the indicated nonradioactive competitor oligonucleotide, and the resulting complexes were separated on a polyacrylamide gel. An arrowhead points to a non-specific complex with unprogrammed reticulocyte lysate. mTRE1, mTRE2, and mTRE3 indicate mutated versions of MMP-9TH TRE1, TRE2, and TRE3, respectively.

Table

Table 1. Comparison of TRE sequences.

TRE sequences of Xl MMP-9TH are aligned with an optimized TRE sequence (Umesono *et al.* 1991) and other *Xenopus* and mammalian TREs. The positions of TREs relative to the transcription start sites are shown. The AGGTCA-like motifs of the DR4 are in capital letters, and bold nucleotides indicate identity with the consensus motif. The intervening letters are in lowercase. xTRβ, TH/bZIP, RMMP-1, rGH, rME, and rMHC indicate *Xenopus laevis* thyroid hormone receptor β (Wong *et al.* 1998), basic region leucine zipper transcription factor (Furlow & Brown 1999), *Rana catesbeiana* MMP-1 (Sawada *et al.* 2001), rat growth hormone (Koenig *et al.* 1987), rat malic enzyme (Jeannin *et al.* 1998), and rat myosin heavy chain (Brent *et al.* 1992), respectively. 'n' in the optimized sequence means any nucleotide.

Gene	location	TREs	Identity with DR4
DR4 TRE		**AGGTCA**nnnn**AGGTCA**	
Xl MMP-9TH TRE1	+760 to +745	**AGGT**T**A**cagc**AGGT**A**A**	10/12
Xl MMP-9TH TRE2	+786 to +771	**AGGT**A**A**gaca**AGG**A**CA**	10/12
Xl MMP-9TH TRE3	-3296 to -3311	**AGGT**A**A**acta**AGG**A**CA**	10/12
xTRβ TRE	+262 to +277	**AGGTCA**tttc**AGG**A**CA**	11/12
TH/bZIP TRE1	-99 to -84	G**GGT**T**A**agta**AGGT**G**A**	9/12
TH/bZIP TRE2	-63 to -78	**AG**T**TCA**aatg**AGG**CTG	8/12
xBTEB TRE	~ -6500	**AG**T**TCA**tctg**AGG**A**CA**	10/12
RMMP-1 TRE	-891 to -876	**AGGT**A**A**gaac**AGG**A**T**A	9/12
rGH TRE	-189 to -174	**AGGT**A**A**gatc**AGG**GAC	8/12
rME TRE	-278 to -263	G**GGT**T**A**gggg**AGG**A**CA**	9/12
rMHC TRE	-134 to -149	**AGGT**G**A**cagg**AGG**A**CA**	10/12

A

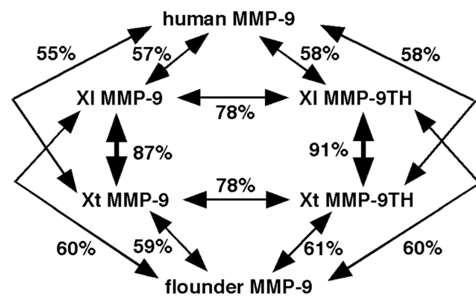

Figure 1

B

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                                      Pre-peptide                        Pro-domain
Xl MMP-9TH   MGGLCVFVLVSILCAWGHSAPTASKTPVSITFPGEIRKNMTSVEVAEWYLVKFGYLPLQQG-SNHHVSLKKALSQMQSKL   79
Xt MMP-9TH   MGWLGILILVAILCSMGHSAPTASKSPVSVIFPGEIRSNMTSVEVAEWYLVKFGYLPLQQGPSNHHVSIKKALSQMQRKL   80
Xl MMP-9     MGRVGVLVLVTILCSKGHSVPIASKSPLTILFPGDIQSGTTDMELAESYLLQFGYLIQEQG-SN--ATLQNALTIMQQKL   77
Xt MMP-9     MGELGVLAIVAILCSRGHSVPTNSKTPLSITFPGEIQSSMTNAELAERYLLQFGYMTQEQG-SN--VTLKNALTLMQQKL   77

Xl MMP-9TH   GLKVTGNLDAETLDAMKTPRCAVPDIGNYNTFEGELKWDHNDLTYRILNYSPDLDPDVIDDAFARAFKVWSDVTPLTFTR   159
Xt MMP-9TH   GLKVTGNLDAETLEAMKSPRCGVPDIGNFNTFDGELKWDHHDITYRILNYSPDLDPDVIDDAFARAFKVWSDVTPLTFTR   160
Xl MMP-9     GLKETGVLDAETLEAMKRPRCGVPDIGQFNTFEGDLKWDHNDITYRILNYSPDLDPEVIDDAFARAFKVWSDVTPLSFTR   157
Xt MMP-9     GLTRTGVLDTETLEAMRRPRCGFPDIGKFNTFDGDLKWDHNDITYRILSYSPDLDPEVIDDAFARAFKVWSDVTPLTFTR   157

                                      Catalytic domain
Xl MMP-9TH   IYSGEPDINIMFGTENHGDPYPFDGKDGLLAHAYPPGPGMQGDAHFDDDEFWTLGTGIVVKTRFGNAEGAACHFPFVFDG   239
Xt MMP-9TH   IYNGEPDINILFGTEDHGDPYPFDGKDGLLAHAYPPGPGMQGDAHFDDDEFWTLGTGTVVKTRFGNAGGAQCHFPFVFDG   240
Xl MMP-9     IYSGEPDINILFGTEDHGDPYPFDRKDGLLAHAYPPGPGVQGDAHFDDDEFWTLGTGTVVKTRFGNAEGALCRFPFTFDG   237
Xt MMP-9     IYSGEPDINILFGPEDHGDPYPFDGKDGLLAHAYPPGPGVQGDAHFDEDEFWTLGTGTVVKTRFGNAEGALCHFPFIFDG   237

             Fibronectin type II repeat            Fibronectin type II repeat
Xl MMP-9TH   QSYNSCTSDGRSDGLPWCSTTPDFDQDKKYGFCPSELLYTYGGNSNGEPCVFPFIFDGVSYKGCTKDGRQDGYRWCSTTA   319
Xt MMP-9TH   QSYNSCTSDGRSDGLPWCSTTPNFDQDKKYGFCPSELLYTYGGNSDGEPCTFPFIFDGVSYDGCTKDGRQDGYRWCGTTA   320
Xl MMP-9     QSFSTCTTAGRSDGLPWCSTTPNYDQDKKYGFCPSEMLYTYGGNSNGQPCVLPFIFDGVSYNGCTKEGRQDGYRWCSTTA   317
Xt MMP-9     QSYSSCTTSGRSDGLPWCSTTPSYDQDKKYGFCPSELLYTYGGNSEGQPCVFPFIFDGVSYNGCTKEGRQDGYRWCSTTA   317

                                 Fibronectin type II repeat
Xl MMP-9TH   NYDQDHKYGFCPNRDTSVIGGNSQGDPCVFPFTFLGKRYNSCTSEGRGDRKLWCATTSSYDQDKKWGFCPDQGYSLFLVA   399
Xt MMP-9TH   NYDQDHKYGFCPNRDISVIGGNSQGDPCVFPFTFLGKRYNSCTSEGRSDRKLWCATTSSYDRDKKWGFCPDQGYSLFLVA   400
Xl MMP-9     NFDQDKKYGFCPNRDTSVIGGNSQGEPCVFPFTFLGKIHNSCTTDGRDDRKLWCATTSNYDLDSKWGFCPDQGYSLFLVA   397
Xt MMP-9     DYDQDQKYGFCPNRDTAVIGGNSQGEPCVFPFTFQGKRFNSCTTDGRDDRKLWCATTSSYDQDRKWGFCPDQGYSLFLVA   397

                    Catalytic domain                                  Hinge region
Xl MMP-9TH   AHEFGHSLGLEHSDVKDALMYPMYSYVKDFELHEDDVNGIQYLYGSG----PHPAPPKPTDKPIPTTT-PSTSITTTTPS   474
Xt MMP-9TH   AHEFGHALGLEHSDVKDALMYPMYSYVKDFQLHEDDVRGIQYLYGSG----PHPAPPKPSDKPIPTTTTPSTRTPTTTPS   476
Xl MMP-9     AHEFGHALGLDHSDVQDALMYPMYSYVKDFQLHQDDVRGIQFLYGTGSGAPPNPNPPKPTKKPLATK-------------   464
Xt MMP-9     AHEFGHALGLEHSEVQDALMYPMYSYVKDFQLHEDDVRGIQYLYGTGS--RPNPNPPKSTKKPLATKS------------   463

Xl MMP-9TH   TRTTTTTPLTPSVNPALDACKVKMFDAIAELQGALHFFKDGLFWTVTSKNKNAPQSPRKISDTWPALPSKIDTAFQDPTS   554
Xt MMP-9TH   TRAPTTTPLTPSVNPALDACKVKMFDAITELQGALHFFKDGLYWSVTSKNKNALQSPRNISDTWPALPTKIDTAFQDPTS   556
Xl MMP-9     -RPRTTTRPVPPVNPAQDACNVEMFDAIADLQGALHFFKDGLYWTLTPRSKNSPQSPLRISDTWPALPSKIDTAFQDPTS   543
Xt MMP-9     -PRRTTTRPVRPVDPAQDACKVEKFDAIADLQGALHFFKDGLYWTLTPRSKNAPQNPQRISDTWPGLPSRIDTAFQDPTS   542

                             Hemopexin/Vitronectin domain
Xl MMP-9TH   KNMFFFSGRKFWQYTGKSVLGPRSIEKLGLSKDVEGIMGSFARDNGKALLFNGERYWRLNVKTLTIDKGYPRLTDVDYAG   634
Xt MMP-9TH   KNIFFFSGRKFWQYTGKSVLGPRSIEKLGLSKDVEGIMGSFARDNGKALLFNGERYWRLNVKTLTVDKGYPRLTDVDYAG   636
Xl MMP-9     KSIFFFSGSKFWQYTGTSAIGPRSIEKLGLSKDVEAIMGSFARDNGKALLFNGEQYWRLNLKTLTIDNGYPRQTADNYPG   623
Xt MMP-9     KSIFFFSGLKFWQYTATSALGPRSIEKLGLNKDVESIMGSFARDNGKALLFNGERYWRLNLKTLTIDNGYPRQTAADYPG   622

Xl MMP-9TH   VPSDSHDVFLYQGKYYFCQDRFFWRMTSRKQVDRVGYVKYDLLHCPEQK   683
Xt MMP-9TH   VPSDSHDVFLYQGKYYFCQDRFFWRMTSRKQVDRVSYVKYDLLHCPEH    684
Xl MMP-9     VPGDSHDVFLYQGNYYFCQDQYFWRVTSRKQQDMVGYVSYDLLRCPQN    671
Xt MMP-9     VPGDSHDVFLYQGNYYFCQDQYFWRVTSRKQQDMVGYVSYDLLHCPQN    670
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C

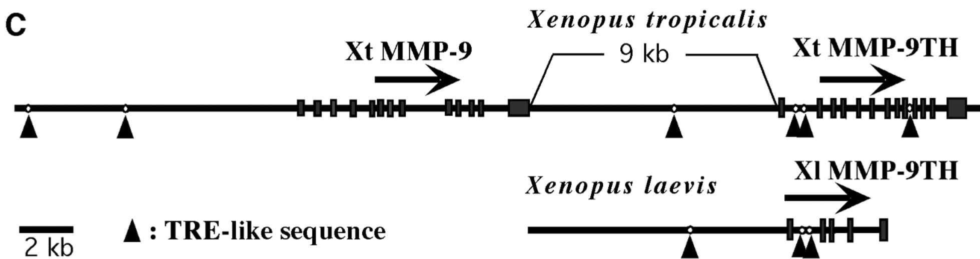
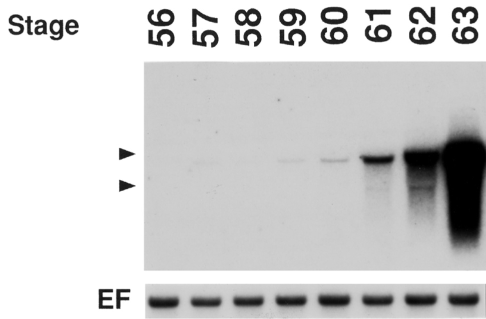


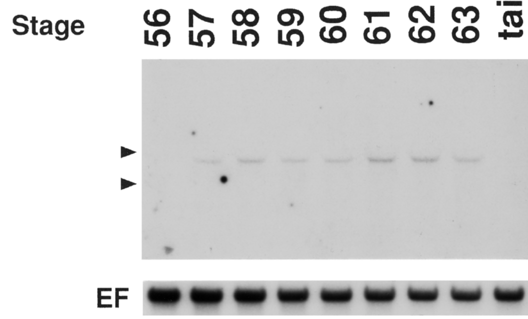
Figure 2

A

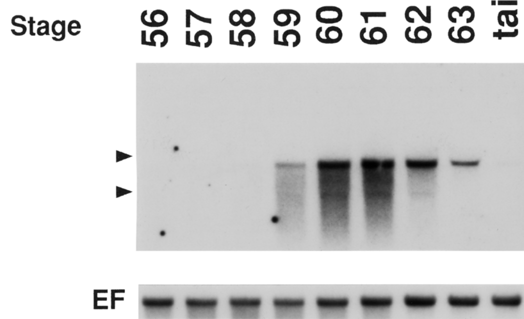
a. Tail



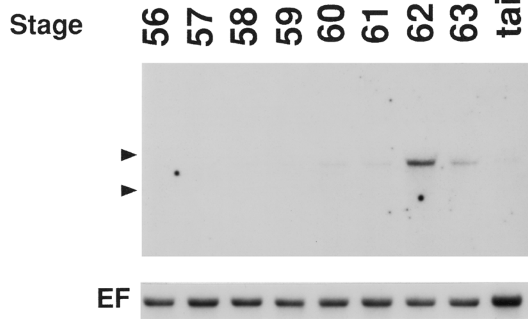
b. Hind Limb



c. Intestine



d. Brain & Spinal Cord



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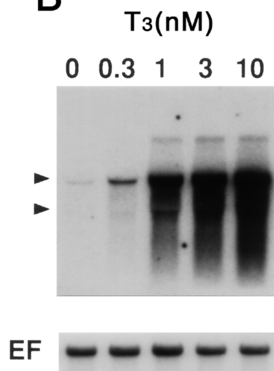

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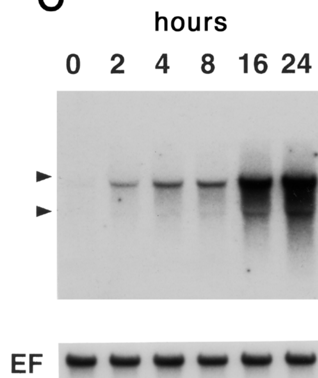

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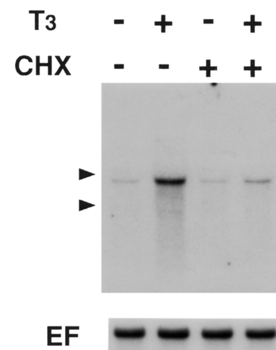
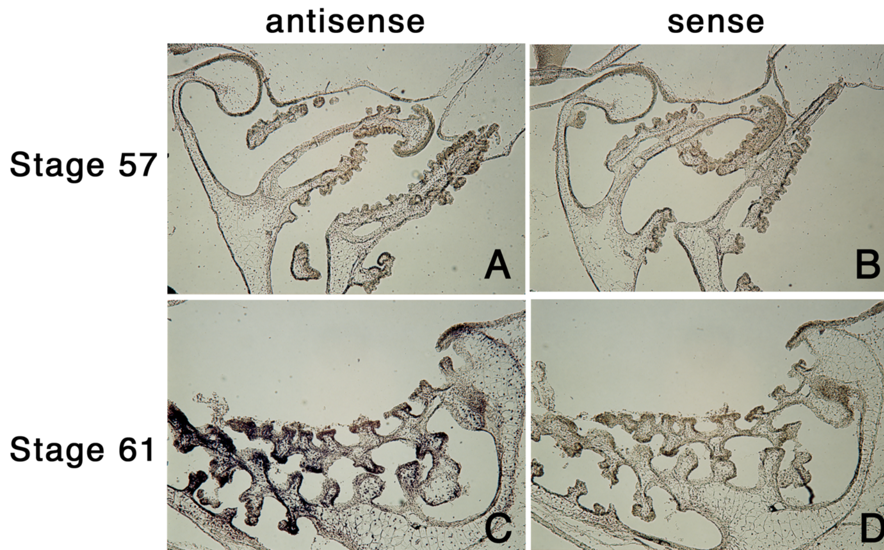


Figure 3



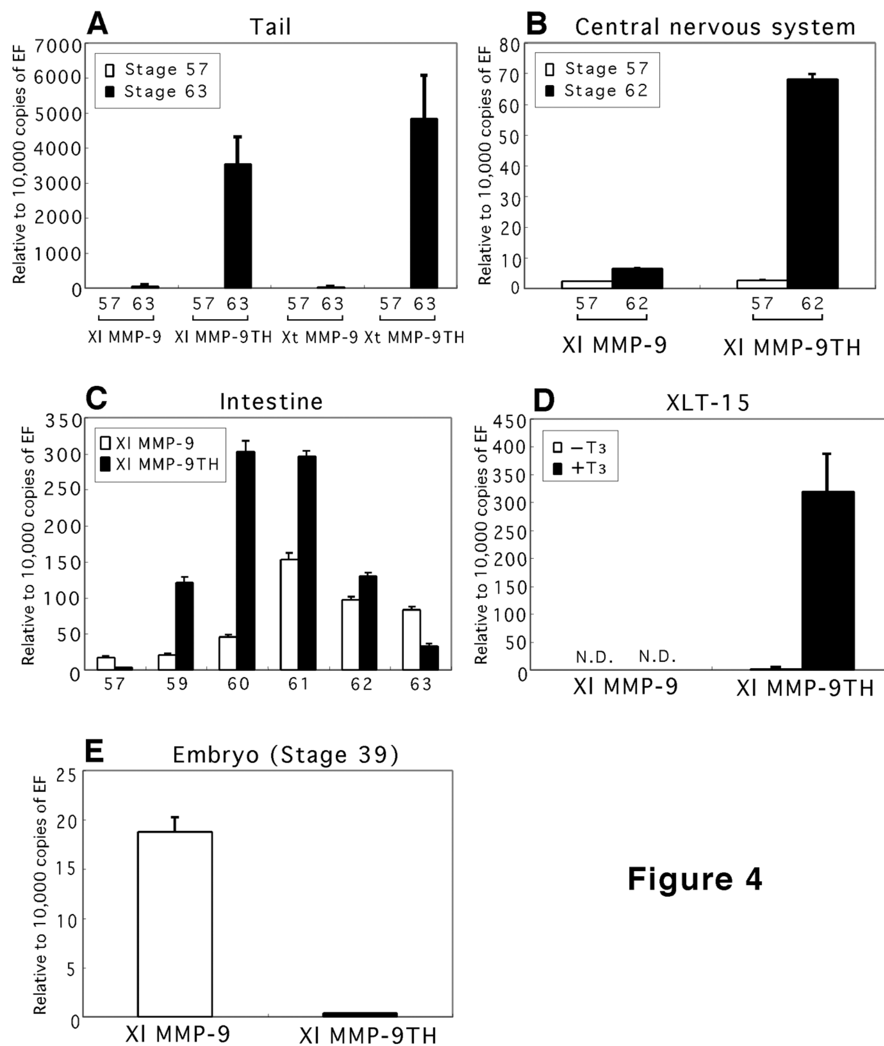


Figure 4

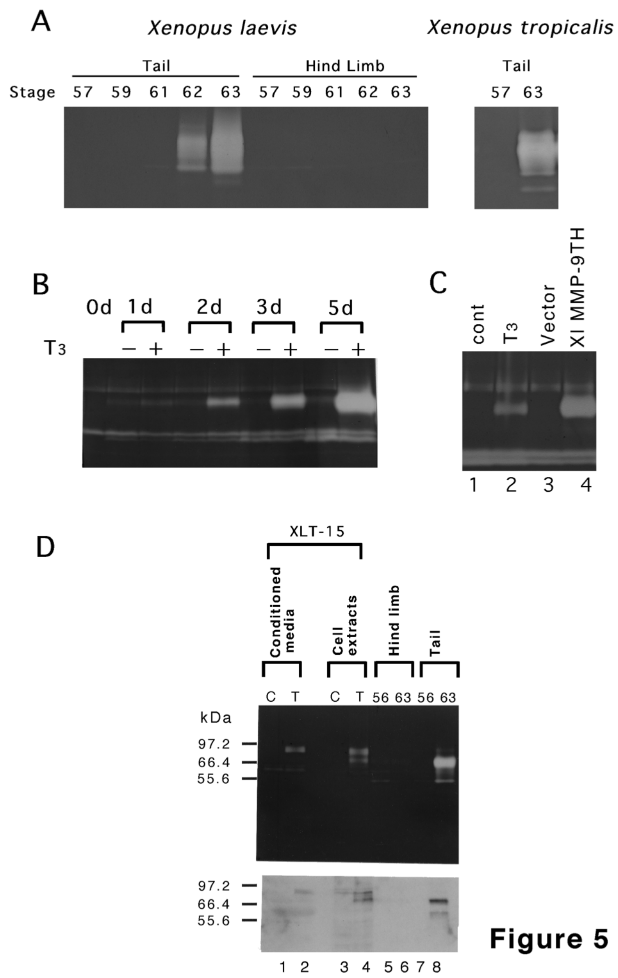
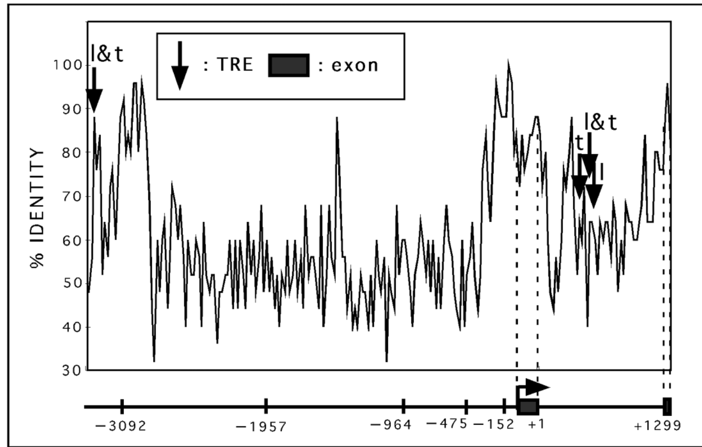


Figure 5

Figure 6

A



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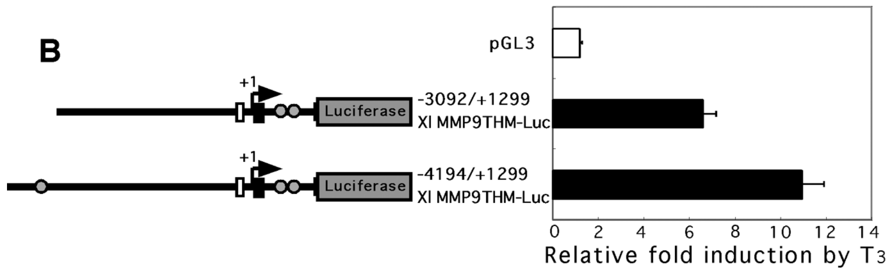

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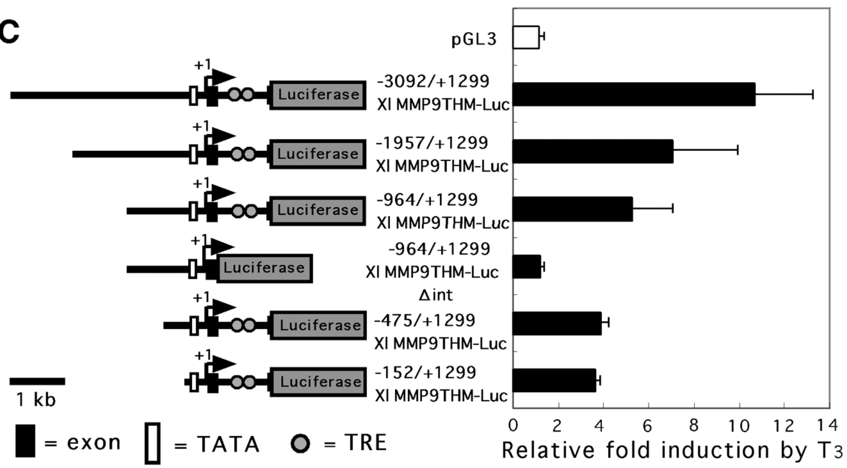

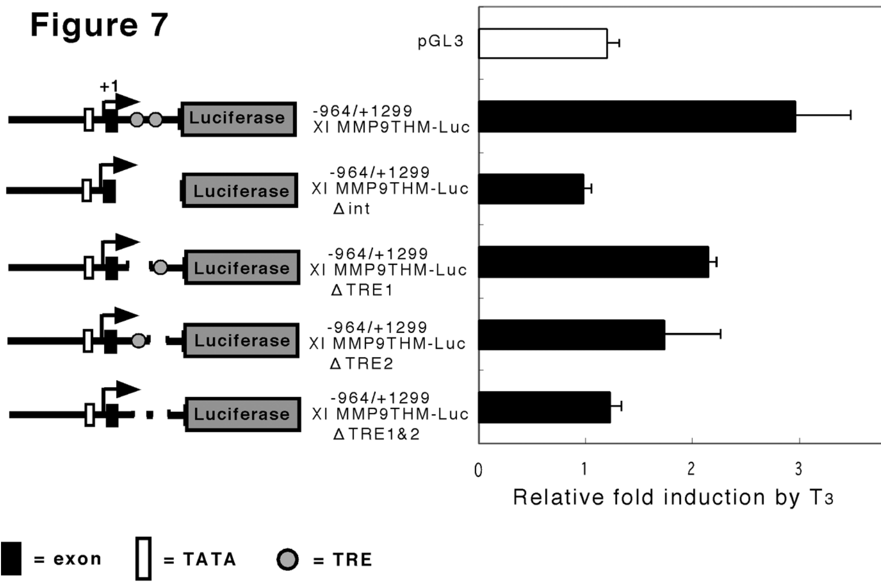

Figure 8