

Thyroid hormone response element-like sequence in anuran matrix metalloproteinase 1 gene is responsive to *in vivo* thyroid hormone administration

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Abstract

Gene (*Rmmp1*) of matrix metalloproteinase 1 (MMP1) of the bullfrog, *Rana catesbeiana*, has been shown previously to contain a thyroid hormone response element (TRE)-like sequence in its 5'-upstream region. The present study aimed to determine whether this TRE-like sequence is functional *in vivo* as a true TRE, and to characterize the sequences of the 5'-upstream region with respect to the regulation of the activity of the TRE when the TRE-like sequence was proved to be a true TRE. With this aim, various sequences of TRE-like sequence-containing 5'-upstream region were constructed and fused to the enhanced green fluorescent protein gene as a reporter gene. The fusion constructs were bombarded to the skin of

bullfrog tadpoles and the activity of the TRE was quantitatively determined by measuring increased intensities of fluorescence when the animals were exposed to thyroid hormone. The present study clearly demonstrated that the sequence of *Rmmp1* is a biologically active TRE *in vivo*. In addition, a unique 36 bp long sequence directly flanked to the 3'-end of the TRE was identified which worked co-operatively with TRE to regulate the transcriptional promoter activity. It should be emphasized that the presence of TRE in the *Rmmp1* gene is unique, because its presence has not been reported in the known promoter region of vertebrate MMPs.

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Introduction

Anuran larvae contain in their life cycle a unique post-embryonic developmental process, metamorphosis, in which they extensively remodel their tissues and transform the body structure into the adult one (Yoshizato & Frieden 1975, Niki *et al.* 1984, Yoshizato 1986, 1989, 1992, 1996, 1998, Oofusa & Yoshizato 1991, Shi 2000). The remodeling consists of two processes which proceed concurrently in a tissue, the breakdown of the larval elements, and the genesis and development of the adult elements (Yoshizato 1986, 1989, 1992, Izutsu *et al.* 1993, Kawai *et al.* 1994, Mukai *et al.* 1995). These two quite different processes take place under the control of the same hormone, thyroid hormone (TH). The former process has attracted researchers because it is expected that a complex but well-organized series of the regulation of the expression of genes is involved in the demolition of tissue macromolecules such as DNA and proteins. In fact, several macromolecule-degrading enzymes and their genes have

been investigated in relation to the breakdown of tissues, such as cathepsin D (Weber 1967, Mukai *et al.* 1995), matrix metalloproteinase (MMP) 1 (Gross & Lapiere 1962, Oofusa *et al.* 1994), MMP11 (Patterson *et al.* 1995), MMP13 (Damjanovski 1999) and MMP18 (Stolow 1996).

Recently, the understanding of anuran metamorphosis at the molecular level has been much increased in relation to the mechanism of regulation of expression of TH-responsive genes (Furlow & Brown 1999, Tata 1999). Among such genes, thyroid hormone receptor (TR) gene has been best characterized in its expression during metamorphosis, in terms of promoter structures, and activators and suppressors acting on the promoter (Glass *et al.* 1989). It has been well established that 3,3',5'-tri-iodothyronine (T₃), an active form of TH, acts on the TH-responsive gene by first binding to the heterodimers of TR and retinoid X receptor (RXR) and the resulting complex of T₃-TR/RXR then binds to a specific sequence called thyroid hormone response element (TRE) present in the 5'-upstream region of the gene (Yu *et al.* 1991).

Gross & Lapiere (1962) discovered collagenase (MMP1) in the tadpoles of the bullfrog, *Rana catesbeiana*, and this plays an essential role in the remodeling of the larval skin. We prepared specific antisera against *Rana* MMP1 and cloned its cDNA (*Rmmp1*) utilizing these antisera. The expression of MMP1 protein and *Rmmp1* was greatly enhanced in bullfrog tadpoles during both T_3 -induced and spontaneous metamorphosis (Oofusa & Yoshizato 1991, Oofusa *et al.* 1994). These studies support the notion that MMP1 is responsible for the extensive remodeling of anuran larval tissues taking place during metamorphosis (Yoshizato 1989). The sequence of the 5'-upstream region of *Rmmp1* revealed the presence of the TRE-like sequence, 5'-AGGTAAGAACAGGATA-3' at -891 to -876 (Oofusa & Yoshizato 1996), which satisfied a general criterion for the identification of TRE proposed by Umesono *et al.* (1991). The binding of the complex of T_3 and TR β /RXR to the TRE-like sequence-containing promoter of *Rmmp1* gene was verified by the gel mobility shift assay and the super shift assay (Oofusa & Yoshizato 1996). Only the region of -891 to -876 (the 'TRE'-like sequence region) of *Rmmp1* in the sequence from -1078 to +98 of *Rmmp1* was shown to be capable of binding to TR by the gel mobility shift assay. However, the biological significance of this TRE-like sequence has been uncertain for the following two reasons. Firstly, the functional *in vivo* assay of this specific sequence has not been done hitherto. Secondly, *Rmmp1* is unique in that there are no other known genes of MMPs that contain the TRE sequence in their transcription-regulatory regions. Thus, it seems to be biologically important to determine whether this unique TRE-like sequence found in amphibia actually plays the role claimed *in vivo*.

These considerations led us to investigate the *in vivo* functions of the TRE-like sequence present in *Rmmp1*. With this aim, we prepared a reporter construct containing enhanced green fluorescent protein (EGFP) gene and a TRE-like sequence-containing the 5'-upstream region of *Rmmp1*. The reporter construct was transfected into tadpoles by a gold particle-mediated gene transfer system (gene gun) and the activity of the reporter gene was quantitatively determined by measuring the change in the intensity of fluorescence in the transfected tissue after treating the animals with T_3 . In the present study, we demonstrated that the TRE-like sequence of *Rmmp1* is biologically active *in vivo* and is a true TRE. In addition, we could identify a unique 36 bp sequence in the 5'-upstream region which regulates TRE activity and is necessary for the T_3 -dependent expression of *Rmmp1*.

Materials and Methods

Animals

Bullfrog tadpoles were purchased from a local animal supplier and staged according to Taylor & Kollros (1946).

The animals were raised in dechlorinated water at 24 °C, and fed boiled spinach. Animals were bombarded and killed in anesthetized conditions by keeping them in iced water.

Plasmid constructs

A 5'-upstream region up to -1078 of *Rmmp1* was cloned as TcgM718 from a bullfrog genomic library constructed in λ FIXII vector (Stratagene, La Jolla, CA, USA) as previously reported (Oofusa & Yoshizato 1996). From TcgM718 a genomic DNA fragment (-1078 to +98) was excised and subcloned into pBSKII(+) (Stratagene) and used as a 'wild' fragment (*Rmmp1*-1078). The wild fragment contained a TRE-like sequence at -891 to -876. Eight 'mutated' fragments (*Rmmp1*-840, *Rmmp1*-524, *Rmmp1*-216, *Rmmp1*-99, *Rmmp1*-TRE840, *Rmmp1*-TRE99, *Rmmp1*-TREf99 and TREf) were generated from this clone (Fig. 1). *Rmmp1*-1078 was digested by *Eco*RI to remove the TRE-like sequence-containing fragment. The fragment thus obtained was *Rmmp1*-840 which did not contain the TRE-like sequence, but contained a sequence from -840 to +98 of the wild fragment. This *Rmmp1*-840 was digested by *Exo*III/MB nuclease (Sambrook *et al.* 1989) to obtain *Rmmp1*-524, *Rmmp1*-216, and *Rmmp1*-99 which also did not contain the TRE-like sequence but contained the basic common promoter sequences of AP-1 and Sp1 (Fig. 1A). *Rmmp1*-840 and *Rmmp1*-99 were ligated to the synthetic double-stranded oligonucleotides of the TRE-like sequence (-891 to -876), which produced *Rmmp1*-TRE840 and *Rmmp1*-TRE99 respectively (Fig. 1B). TREf was a synthetic oligonucleotide consisting of TRE-like sequence and its 3'-flanking 36 bp sequence (-875 to -840). *Rmmp1*-99 was ligated to TREf to make *Rmmp1*-TREf99. These wild and mutated fragments were inserted into pEGFP-1 (Clontech, Palo Alto, CA, USA) as shown in Fig. 1A and B. As a result we obtained the following nine fragments fused to the EGFP gene: (1) pEGFP/*Rmmp1*-1078, (2) pEGFP/*Rmmp1*-840, (3) pEGFP/*Rmmp1*-524, (4) pEGFP/*Rmmp1* TRE-216 and (5) pEGFP/*Rmmp1*-99 as shown in Fig. 1A, and (1) pEGFP/*Rmmp1*-TRE840, (2) pEGFP/*Rmmp1*-TRE99, (3) pEGFP/*Rmmp1*-TREf99 and (4) pEGFP/TREf as shown in Fig. 1B.

A plasmid containing a 5'-flanking region (-1562 to +312) of xTR β A1 gene (pEGFP/TR β A1) (Ranjan *et al.* 1994) was used as a positive control construct for TH-responsive expression of the EGFP gene. Red fluorescent protein (RFP) gene was fused to the human cytomegalovirus (CMV) promoter as follows. A plasmid containing the CMV-IE94 promoter (pRc/CMV) was purchased from Invitrogen (Carlsbad, CA, USA). A *Bgl*II-*Hind*III fragment of pRc/CMV containing an essential promoter region was subcloned into pDsRed1-1 (pDsRed1/CMV) which contained the open reading

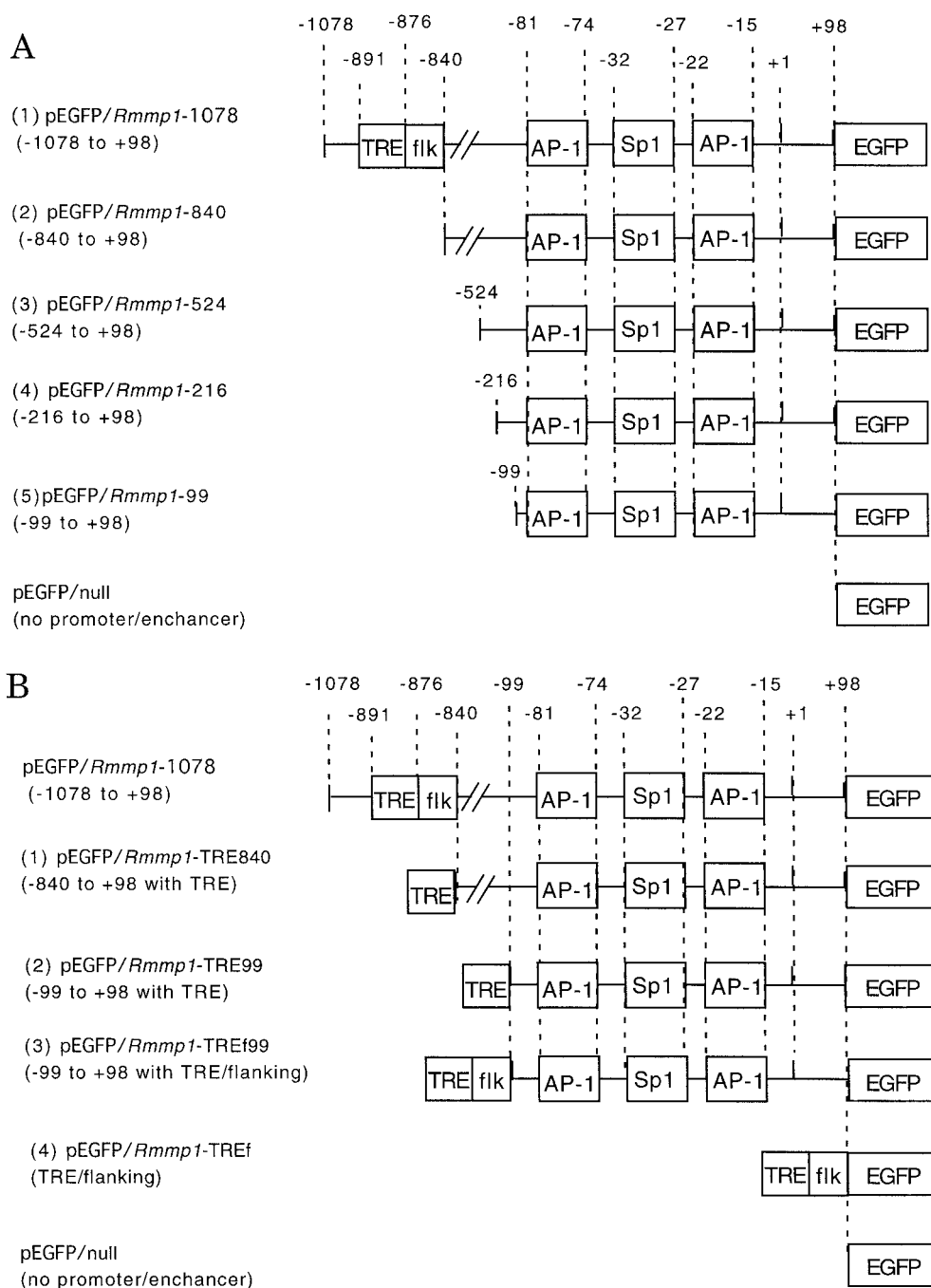


Figure 1 Schematic drawings of constructs of fusion genes. (A) Fusion genes without the TRE-like sequence. (1) *Rmmp1*-1078; the 5'-upstream region from -1078 to $+98$ contained a TRE-like sequence (TRE) at -891 to -876 , and potential binding sites of transcription factors, two AP-1 at -81 to -74 and -22 to -15 , and Sp1 at -32 to -27 . Four shorter constructs were made from this construct by deleting it with *EcoRI* and *ExoIII*/MB nuclease: (2) *Rmmp1*-840 (-840 to $+98$), (3) *Rmmp1*-524 (-524 to $+98$), (4) *Rmmp1*-216 (-216 to $+98$) and (5) *Rmmp1*-99 (-99 to $+98$). These constructs were fused to EGFP gene producing (1) pEGFP/*Rmmp1*-1078, (2) pEGFP/*Rmmp1*-840, (3) pEGFP/*Rmmp1*-524, (4) pEGFP/*Rmmp1*-216 and (5) pEGFP/*Rmmp1*-99. 'flk' is the 36 bp long (-875 to -840) 3'-flanking region of the TRE-like sequence. pEGFP/null was a construct without any of these sequences of the region. (B) Deletion genes with the TRE-like sequence. *Rmmp1*-TRE840 (construct 1) was obtained by fusing *Rmmp1*-840 to the synthetic TRE sequence. Ligation of the TRE with *Rmmp1*-99 produced *Rmmp1*-TRE99 (construct 2). *Rmmp1*-TREf99 (construct 3) was obtained by ligating TREf to *Rmmp1*-99. TREf (construct 4) was a synthetic TRE and its 3'-flanking region from -875 to -840 . These constructs were fused to EGFP gene producing (1) pEGFP/*Rmmp1*-TRE840, (2) pEGFP/*Rmmp1*-TRE99, (3) pEGFP/*Rmmp1*-TREf99 and (4) pEGFP/TREf.

frame of RFP (Clontech). We confirmed that pDsRed1/CMV did not respond to T_3 by the *in vivo* assay method described below and it was used as an internal standard to normalize the level of expression of each tested construct among experimental animals throughout this study. These plasmids were propagated by a standard protocol and purified using a Wizard Plasmid Purification System (Promega, Madison, WI, USA).

The T_3 responsiveness of the above constructs fused to the enhanced EGFP gene was quantitatively determined *in vivo* by utilizing a dual reporter gene system. These constructs were bombarded to tadpoles together with the construct of pDsRed1/CMV for the 'fluorescent dual reporter system'. An appropriate ratio of constructs of EGFP and RFP for a better discrimination between green (EGFP) and red (RFP) signals was found to be 3:1. Plasmid constructs (45 μ g) to be tested and 15 μ g pDsRed1/CMV as the internal control were mixed and were co-precipitated with 25 mg spherical gold particles (1.0 μ m diameter; Bio-Rad Laboratories, Hercules, CA, USA). The precipitates were suspended in 3 ml solution of 10 μ g/ml polyvinylpyrrolidone (Bio-Rad Laboratories) in ethanol (liquid chromatograph grade; Nacalai Tesque, Kyoto, Japan) and coated to the inner wall of a 63.5 cm long GoldCoat tubing (Bio-Rad Laboratories) according to the manufacturer's protocol. This tube was evenly cut into fifty 1.27 cm long cartridges. One piece of these cartilages containing 1.2 μ g DNA was used for a bombardment to the back or tail skin of a tadpole.

Gene transfer and TH treatment

Tadpoles at stages X to XII were bombarded with the plasmids as described above at appropriate sites of the back skin or the lateral side of the tail skin using a gold particle-mediated gene transfer system (HERIOS Gene Gun; Bio-Rad Laboratories). Our preliminary tests to optimize the procedure of the gene transfer in terms of the transfer efficiency showed that the firing pressure was best at 1000 kPa (150 pounds per square inch). We bombarded a tadpole with 0.5 mg gold particles at each shot. A better gene transfer efficiency was obtained for the DNA loading ratio of 1.2 μ g plasmid DNA to 0.5 mg gold particles for a bombardment. For the accurate estimation of the expression level of EGFP and RFP genes by photographing test animals and the following computer-aided processing of the images which are described below, it was necessary to transfer the reporter construct to the outermost layer of the epidermis as uniformly as possible. We confirmed that the above-described conditions of gene transfer using a gun satisfied this requirement. The fluorescent signal of EGFP began to be seen at 16 h after the bombardment whereas that of RFP was seen at 30 h after the bombardment. These two fluorescent signals continued to be stably expressed up to 120 h. We measured the expression of the reporter construct of pEGFP/*Rmmp1*-1078 at 0, 1, 5 and

10 nM T_3 (Sigma, St Louis, MO, USA). The expression increased dose-dependently up to 5 nM, where the expression reached a plateau level (data not shown). Therefore, in the present study we usually treated tadpoles at a dose of 5 nM. T_3 treatment was started at 48 h after the bombardment when necessary.

Fluorescent observations

Animals were anesthetized in iced water for several minutes, photographed by a fluorescent dissecting microscope (MZ FLIII; Leica, Bensheim, Germany) equipped with a pertainer chilled color charge-coupled device (CCD) camera (C5810; Hamamatsu Photonics, Hamamatsu, Japan), a personal computer (PowerMacintosh G3; Apple Computer, Cupertino, CA, USA), and a high-grade printer system (EPSON, Suwa, Japan). The 'GFP' filter was used to observe the EGFP expression and the 'G' (rhodamine) filter for the RFP expression. Using this system, the 'G' filter eliminated all of the green signals. On the other hand, the 'GFP' filter could not cut off all of red signals, about 15% of RFP fluorescence contaminating the green signal. The digital camera we used for this study has three CCD chips allowing channel separation among the three primary colors. For eliminating the 'contaminated' red signals in the GFP observations, photographs were processed by using Photoshop 5.5 (Adobe System, Mountain View, CA, USA) which completely separated red signals from green signals. We were convinced that there was not a photo-bleaching problem in the fluorescence intensity of EGFP and RFP in this study from the results of the following preliminary experiments. Two tadpoles were bombarded with pEGFP/CMV and pDsRed/CMV respectively, and these two tadpoles were exposed to the excitation beam for 5 s for taking fluorescent photographs. For both constructs, such an exposure was repeated five times with 10-s intervals. There was no photo-bleaching in these photoimages (data not shown).

Statistical analysis

Results shown in Fig. 3 are presented as the mean \pm s.d. and their statistical analysis was performed using a paired *t*-test followed by Fisher's test to check the equivalence of the variances.

Results

We examined whether the reporter construct of TRE-containing promoter and EGFP gene can be actually expressed *in vivo* in response to TH. Tadpoles at stage X were bombarded with the construct of pEGFP/TR β A1 as a positive control gene to demonstrate the validity of the gold particle-mediated gene transfer system as a method of gene transfer for tadpoles. The gene was shot at regions of

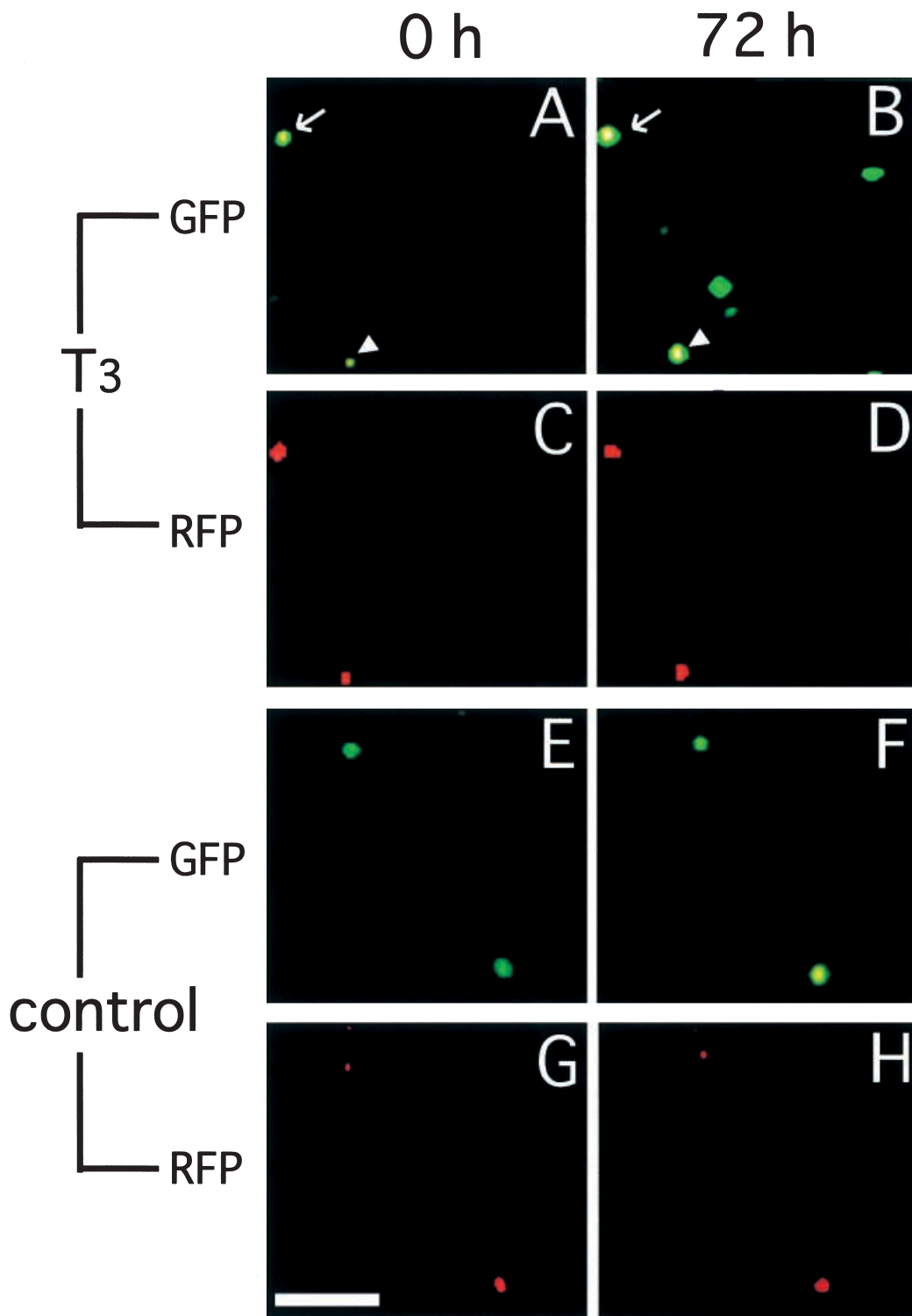


Figure 2 Responsiveness of pEGFP/Rmmp1–1078 to T_3 . The mixture of two constructs, pEGFP/Rmmp1–1078 and pDsRed1/CMV, was bombarded to the tail skin of stage X tadpoles. Two days later tadpoles were placed in water with (A, B, C, D) and without 5 nM T_3 (E, F, G, H). Tadpoles were seen through a green filter (A, B, E, F) and a red filter (C, D, G, H) at 0 h (A, C, E, G) and 72 h (B, D, F, H) of T_3 treatment. The spots marked by the arrow and the arrowhead in A and B indicate that they are the same spot (cell) observed at the different time-period of the experiment. Bar=0.25 mm.

the back skin or the lateral side of the tail skin. Two days later the animals were bathed in water with or without 5 nM T_3 . The tadpoles in T_3 -containing water became fluorescent at 24 h of the hormone treatment whereas the tadpoles in control water did not show such changes in fluorescence (data not shown). Identical responses of the fusion gene were seen at the back skin and the tail skin.

We prepared four DNA fragments from a 5'-upstream region of *Rmmp1* gene and made the following five constructs fused with EGFP genes: (1) pEGFP/*Rmmp1*-1078 (wild fragment), (2) pEGFP/*Rmmp1*-840, (3) pEGFP/*Rmmp1*-524, (4) pEGFP/*Rmmp1*-216 and (5) pEGFP/*Rmmp1*-99 (Fig. 1A). To measure the background level of EGFP fluorescence, pEGFP alone (pEGFP/null) was also prepared which did not contain any sequence of the 5'-upstream region of *Rmmp1*. Among these constructs, only pEGFP/*Rmmp1*-1078 contained the TRE-like sequence. Tadpoles at stage X were bombarded with these constructs at the back skin and the lateral side of the tail skin, and placed in water with and without 5 nM T_3 for 72 h. The animals were observed through a green or red filter. The construct of pEGFP/*Rmmp1*-1078 but no other constructs gave a visible enhancement of fluorescent signals when the tadpoles were exposed to T_3 (Fig. 2, compare B with A). Such an enhancement was not seen in T_3 -free tadpoles (Fig. 2E and F). The efficiency of gene transfection appeared to be relatively constant among the tadpoles tested because red-colored spots of RFP were seen with similar frequencies among the tadpoles examined (data not shown). This situation was quantitatively demonstrated as described in Fig. 3 where the intensity of green fluorescence expressed by these constructs was calculated by computer-aided processing of photographs. T_3 elevated sevenfold the fluorescence of *Rmmp1*-1078, but not that of other constructs (Fig. 3A). In addition, similar levels of fluorescence intensity were reproducibly obtained in these quantifications with a quite low variance for each of the tested constructs (Fig. 3), allowing us to quantitatively compare the intensity among the constructs shown in Figs 2 and 3 on a common basis. From these results we concluded that the TRE-like sequence in the 5'-flanking region is a true TRE and is termed TRE sequence hereafter. The response of the construct of pEGFP/*Rmmp1*-1078 was quantified against the length of T_3 treatment by determining the fluorescent intensities with 'Photoshop' (Fig. 4). *Rmmp1*-1078 responded well to T_3 at 24 h, the response continuing to increase thereafter until at least 72 h. The increase was 6.7- and 9.3-fold at 24 h and 72 h respectively. In contrast, *Rmmp1*-1078 without T_3 and pEGFP/null with and without T_3 did not show any change during the experimental period.

The results of the experiments described above showed that *Rmmp1* requires the presence of the TRE sequence to respond to T_3 . We thought about the possibility that other sequences in the 5'-upstream region might regulate the

activity of the TRE to respond to T_3 . To test this, four additional transgenes (*Rmmp1*-TRE840, *Rmmp1*-TRE99, *Rmmp1*-TREf99 and TREf) were constructed by variously deleting sequences of a 3'-flanking region of TRE and -840 to +98 (Fig. 1B).

These constructs were fused to the EGFP gene and were given to tadpoles as above, which were then exposed to 5 nM T_3 for 72 h. The fluorescence intensity was quantified and is shown in Fig. 3B. Among them only the construct of *Rmmp1*-TREf99 significantly responded to T_3 , its expression being enhanced 2.2 times. The *Rmmp1*-TREf99 contained the 3'-flanking region and sequence of -99 to +98. When one of the two sequences was absent as in *Rmmp1*-TRE99 in which the former was absent or TREf in which the latter was absent, the T_3 responsiveness of the TREf was lost, indicating that both regions are together required for TRE to respond to T_3 . The requirement of the region of -99 to +98 was understandable, because this region contained the basic promoters of AP-1 and Sp1 (Fig. 1B) which are known to be necessary for the initiation of the transcription in human gelatinase gene (Huhtala *et al.* 1991).

Figure 3 demonstrates that constructs of *Rmmp1*-524, *Rmmp1*-216, *Rmmp1*-99 and *Rmmp1*-TRE840 showed a higher expression at both 0 and 72 h than the construct of *Rmmp1*-1078 at 0 h. If we define the expression of these constructs in the absence of T_3 as the basic expression, the basic expression of these four constructs was significantly higher than that of constructs of *Rmmp1*-1078, *Rmmp1*-840, *Rmmp1*-TRE99 and *Rmmp1*-TREf99. All these eight constructs commonly contained basic promoters of AP-1 and Sp1. The constructs of *Rmmp1*-1078 and *Rmmp1*-840 contained a sequence of -840 to -524. Thus, this region seemed to suppress the basic expression induced by AP-1 and Sp1 to a level of the expression without AP-1 and Sp1. Comparison of the expression between *Rmmp1*-840 and *Rmmp1*-TRE840 also suggested that the TRE itself eliminates the suppressive effect of this region on the basic expression. The low or no basic expression by *Rmmp1*-TRE99 and *Rmmp1*-TREf99 suggested that the TRE sequence suppresses the basic expression. Although the construct *Rmmp1*-TRE840 and *Rmmp1*-TRE99 contained the TRE sequence, these did not respond to T_3 , which again indicated the importance of the 3'-flanking sequence to potentiate the activity of the TRE sequence.

Discussion

MMP1 is suggested to be a typical housekeeping gene. Its major substrate is type I collagen which is the most abundant protein in the vertebrate body and, therefore, the enzyme is required throughout the life cycle of the tadpole to regulate the metabolism of this substrate. We have previously reported the presence of a TRE-like

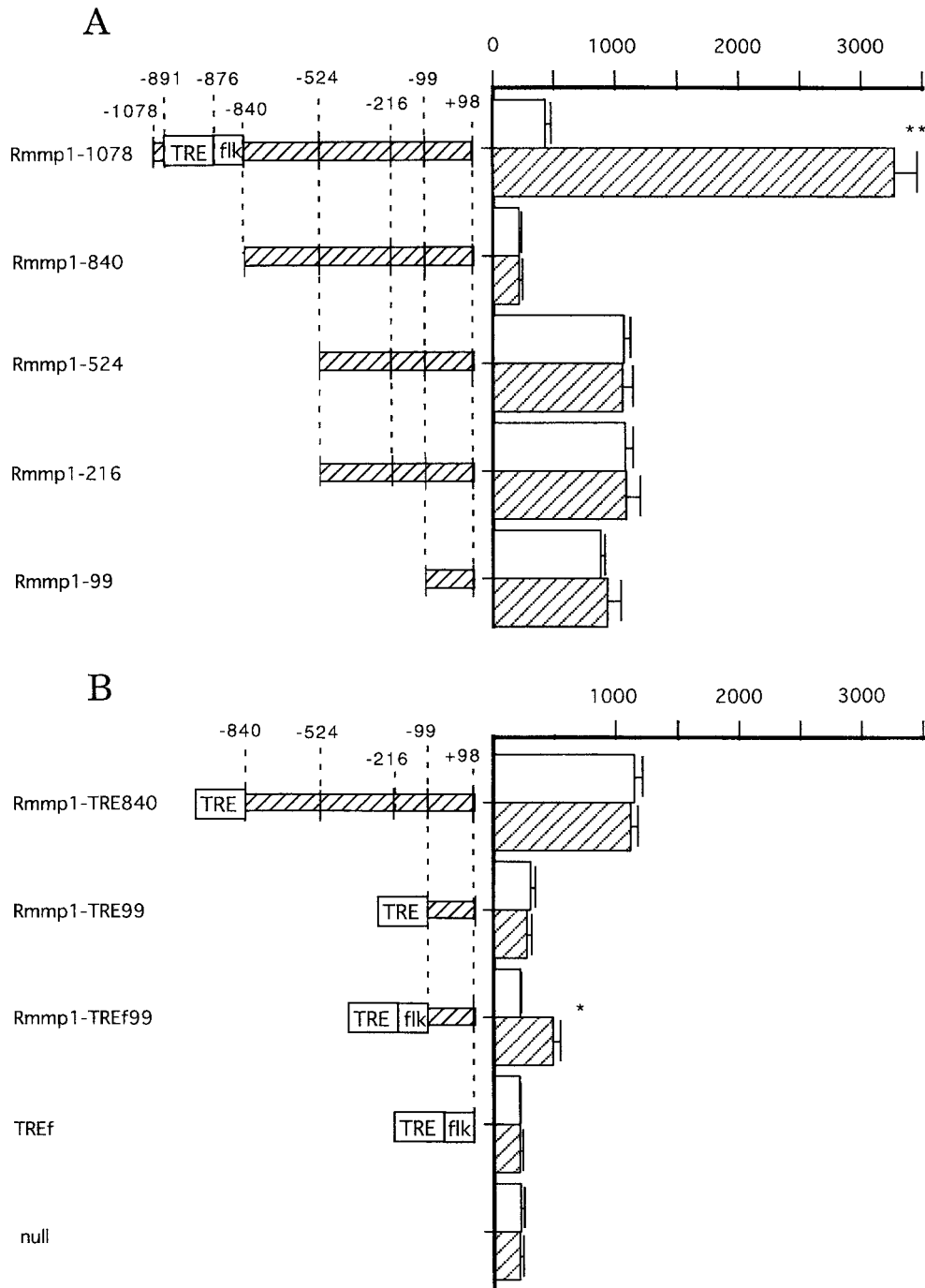


Figure 3 Quantification of the intensity of green fluorescence expressed by the transgenes. Tadpoles were transferred with ten constructs. Two days later, animals were reared for 72 h with (hatched bars) and without (open bars) 5 nM T₃. The constructs of *Rmmp1*-1078 through *Rmmp1*-99 are grouped in (A) and the remaining in (B) for the sake of easy comparison. Values represent the mean of eight independent determinations (eight tadpoles) with lines showing S.E.M. **P*<0.01, ***P*<0.005, statistically significant level between with and without T₃.

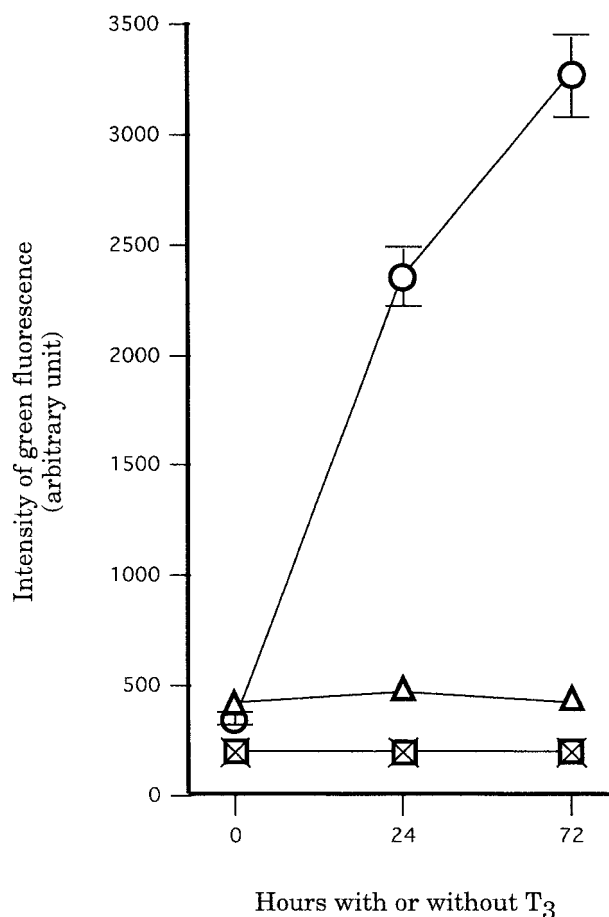


Figure 4 Quantitation of the response of pEGFP/*Rmmp1*-1078 to T₃. Tadpoles were transfected with pEGFP/*Rmmp1*-1078 (○, △) or pEGFP/null (□, ×). Two days later animals were placed in water with (○, □) and without (△, ×) 5 nM T₃ and kept there for 72 h. Fluorescent intensity in the gene-transferred area of the animals was quantified at 0, 24 and 72 h. The intensity of green fluorescence was normalized by dividing it by the intensity of red fluorescence. Each point represents the mean of eight determinations (eight tadpoles) with S.E.M.

sequence in the 5'-upstream region of *Rmmp1* (Oofusa & Yoshizato 1996). This sequence was shown to bind *in vitro* specifically to T₃ in the presence of nuclear extracts prepared from T₃-treated tadpoles. However, it remained to be determined if this TRE-like sequence is a functional TRE. This is an important question to address because, to the best of our knowledge, human MMP1 gene does not contain TRE in its promoter region and, to our knowledge, there are no other known genes of the MMP family reported hitherto which were shown to contain TRE. We asked whether such a housekeeping gene contains TRE in its transcription regulatory region. Up to now a few housekeeping genes have been reported to have a TRE sequence in their transcriptional regulation sequences,

such as malic enzyme (Petty *et al.* 1990) and myosin heavy chain (Flink & Morkin 1990). As shown in Fig. 1, *Rmmp1* gene lacks the TATA box and has an Sp1 (-32 to -27) site adjacent to the putative transcriptional start point (Oofusa & Yoshizato 1996). The human 92 kDa type IV collagenase gene also lacks the TATA box in its flanking region and its Sp1 site plays an important role in the expression of the gene (Huhtala *et al.* 1991). The present study was performed with two aims. One was to determine if the previously reported TRE-like sequence found in *Rmmp1* (Oofusa & Yoshizato 1996) is a true TRE and functional *in vivo*. If the TRE-like sequence was proven to be a true TRE we had the second aim to examine whether the activity of the TRE to respond to T₃ is regulated by some specific sequences present in the 5'-upstream region of *Rmmp1*.

To determine the responsiveness of the TRE-like sequence-containing upstream region to T₃, we constructed a gene by fusing the upstream region to EGFP gene as a reporter gene. To find out whether EGFP is metabolically stable *in vivo* and can be reliably used as a reporter during an experimental period, a reporter construct consisting of a CMV promoter and an EGFP gene was constructed, and bombarded the bullfrog tadpole skin as in the promoter of *Rmmp1*/EGFP gene. The expression was detectable by its fluorescence without any decay in its intensity for at least 1 week (data not shown), ensuring the usability of EGFP as a reporter in living tadpoles. In addition, we utilized a dual reporter system consisting of EGFP and RFP which can be detected through different filters. RFP reporter was used to normalize the intensity of transfected EGFP-containing genes among different tadpoles which were transfected with a different efficiency. With this dual reporter system we could correctly and reliably compare the intensity of EGFP signals among different animals. The computer software 'Photoshop' was able to separate the green signal (EGFP) and the red signal (RFP) from fluorescent signals obtained from one experimental animal, and was able to numerically measure separately each dual fluorescent intensity. To make the quantification of fluorescent signals reliable, we optimized the conditions of bombardment of the fusion genes. The distribution of the transfected cells was examined on frozen section of experimental animals. If fluorescence-positive cells were distributed differently in tissues at different levels of depth from the skin surface, we could not correctly quantify the fluorescent signals in a comparable manner among test animals, because the fluorescent intensity is reduced as the cells are at a deeper location. In fact, all fluorescent signals were found in the cells at the surface of the skin (apical cells): all of 63 positive cells among 65 frozen sections checked were in the outermost apical layer (data not shown).

We prepared nine different constructs which contained different sequences of the 5'-upstream region of *Rmmp1*

and fused them to the EGFP gene. These fusion genes were transferred to the back skin and the tail skin of tadpoles using a gene gun, and the fluorescent intensity was measured. The longest fragment (wild fragment, *Rmmp1*-1078) was shown to respond well to exogenous 5 nM T₃ at 24 and 72 h after the treatment and any other tested fragments without the TRE-like sequence did not show such T₃ responsiveness, clearly demonstrating that the TRE-like sequence is a true TRE.

Rmmp1-1078 was a 5'-upstream region of *Rmmp1* with a part of the first intron covering from -1078 to +98. The *in vivo* expression of this construct without T₃ exposure (basic expression) was relatively low, as compared with that of *Rmmp1*-524, *Rmmp1*-216 and *Rmmp1*-99 which commonly lacked TRE and the sequence of -875 to -840, but the *Rmmp1*-1078 was intensively upregulated when exposed to T₃. These results might be explained by interpreting as follows: (1) TRE-like sequence is a true TRE as concluded above, (2) TRE itself functions as a suppressor when the ligand (T₃) is not present as previously shown by Perlmann *et al.* (1993) and Kurokawa *et al.* (1993), and (3) the sequence of -875 to -840 itself or together with other sequences suppresses the basic expression. However, the last interpretation is not plausible because the basic expression of *Rmmp1*-840, which lacks the sequence of -875 to -840, was rather lower than that of the wild fragment.

The constructs of both *Rmmp1*-TRE840 and *Rmmp1*-TRE99 contained the TRE, but their expression was not sensitive to T₃. Both constructs contained the sequences of -99 to +98 and, therefore, these sequences are suppressive for the function of TRE. These fragments did not commonly contain the sequence of -875 to -840 and, therefore, this 36 bp long direct 3'-flanking sequence was suggested to be necessary for the function of TRE, acting as its activator. This suggestion was supported by the observed expression of *Rmmp1*-TRE99 and *Rmmp1*-TREf99, the latter being a fragment constructed in such a way that the 36 bp sequence was additionally ligated to the former. This addition conferred the responsiveness to T₃ upon the *Rmmp1*-TRE99. In contrast to this role as an activator, the 36 bp sequence also appears to act as a suppressor in the absence of T₃, because the higher basic expression of *Rmmp1*-TRE840 was decreased to a level comparable with that of *Rmmp1*-840. Thus, it seems most likely that the region of -875 to -840 itself or part of it acts co-operatively with TRE to regulate the transcriptional promoter activity. This region did not contain any known consensus sequences which could act as a regulator of gene expression.

The present study clearly demonstrates that *Rmmp1*, a typical housekeeping gene, contains a true TRE in its 5'-upstream promoter region. The human MMP1 gene (accession nos AF023338 and D26110) was confirmed by us not to contain either the direct repeat or the palindrome-type of TRE by surveying the TRE-like

sequence on the published nucleotide sequence. There have been no reports on the structure of the genomic gene of MMP1 of other vertebrates such as birds, reptiles and fishes. The biological significance of the presence of TRE in anuran MMP1 is not clear at present. Our data support the hypothesis that a TRE in anuran MMP1 evolved to regulate the remodeling of larval tissue to adult during metamorphosis (Oofusa & Yoshizato 1991, 1996). Further works are required to determine whether other anuran MMPs contain TRE and if this observation extends to other vertebrates besides humans.

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