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Molecular Characterization of Thyroid Hormone Receptors from the Leopard Gecko, and Their Differential Expression in the Skin

Yoh-Ichiro Kanaho, Daisuke Endo and Min Kyun Park*

Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Tokyo 113-0033, Japan

Thyroid hormones (THs) play crucial roles in various developmental and physiological processes in vertebrates, including squamate reptiles. The effect of THs on shedding frequency is interesting in Squamata, since the effects on lizards are quite the reverse of those in snakes: injection of thyroxine increases shedding frequency in lizards, but decreases it in snakes. However, the mechanism underlying this differential effect remains unclear. To facilitate the investigation of the molecular mechanism of the physiological functions of THs in Squamata, their two specific receptor (TRα and β) cDNAs, which are members of the nuclear hormone receptor superfamily, were cloned from a lizard, the leopard gecko, *Eublepharis macularius*. This is the first molecular cloning of thyroid hormone receptors (TRs) from reptiles. The deduced amino acid sequences showed high identity with those of other species, especially in the C and E/F domains, which are characteristic domains in nuclear hormone receptors. Expression analysis revealed that TRs were widely expressed in many tissues and organs, as in other animals. To analyze their role in the skin, temporal expression analysis was performed by RT-PCR, revealing that the two TRs had opposing expression patterns: TRα was expressed more strongly after than before skin shedding, whereas TRβ was expressed more strongly before than after skin shedding. This provides good evidence that THs play important roles in the skin, and that the roles of their two receptor isoforms are distinct from each other.

Key words: thyroid hormone receptor, TR, skin shedding, reptile, Squamata, leopard gecko

INTRODUCTION

The thyroid hormones (THs), thyroxine (T_4) and thyronine (T_3), are pleiotropic factors important for many developmental and physiological processes in vertebrates. There has been a lot of research into the physiological significance of THs in various vertebrates. For example, THs are known to be important for inner ear and retina development, liver metabolism in mice (Flamant and Samarut, 2003), metamorphosis in axolotl and *Xenopus* (Nakajima *et al*., 2005; Sachs *et al*., 2000; Safi *et al*., 2004), and embryogenesis and metamorphosis in many teleost fish (Power *et al*., 2001).

In reptiles, THs have been suggested to affect tail regeneration (Turner and Tipton, 1971), metabolic rate and metabolic enzyme activity (John-Alder, 1990; John-Alder and Joos, 1991), and shedding frequency (Chiu *et al*., 1967; Chiu and Lynn, 1970). Above all, their effect on shedding frequency is particularly interesting. In lizards, the injection of thyroxine increases shedding frequency, and thyroidectomy decreases it (Chiu *et al*., 1967). In contrast, in snakes, the injection of thyroxine decreases the shedding frequency, and thyroidectomy increases it (Chiu and Lynn, 1970). The mechanisms underlying these completely opposite phenomena have not been clarified, partly due to the lack of investigation of the molecular mechanism of THs in reptiles.

THs can regulate target genes by interacting with thyroid hormone receptors (TRs), which are members of the nuclear receptor superfamily. Two isoforms, TRα and TRβ, have been isolated from species of four classes of vertebrate, but not from reptiles (Forrest *et al*., 1990; Kawakami *et al*., 2003; Murray *et al*., 1988; Yaoita *et al*., 1990). These isoforms share high homology and have similar biochemical properties. However, they have distinct spatial and temporal expression profiles in overlapping patterns, suggesting that two genes mediate both individual and common biological functions.

Unlike other squamate animals, the leopard gecko, *Eublepharis macularius*, is easily maintained and bred in the laboratory. The leopard gecko is therefore expected to become an experimental model. Indeed, several molecular studies of the endocrine system have already been conducted on this species (Endo and Park, 2004; Endo and Park, 2005; Ikemoto and Park, 2003; Ikemoto *et al*., 2004; Kato *et al*., 2005; Valleley *et al*., 2001).

In this study, we cloned TRα and β from the leopard gecko to augment investigations on the molecular mechanisms of the physiological functions of THs in reptiles. In

* Corresponding author. Phone: +81-3-5841-4439;
Fax : +81-3-5841-4439;
E-mail: biopark@biol.s.u-tokyo.ac.jp
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addition to identifying two isoforms of TR, we performed phylogenetic and expression analyses. We also demonstrated the differential expression of the TR isoforms, and herein discuss their possible roles in shedding.

MATERIALS AND METHODS

Animals

The leopard geckos (*Eublepharis macularius*) were treated according to the guidelines of the Bioscience Committee at the University of Tokyo. The animals were provided meal worms, crickets, water, and powdered calcium supplement *ad libitum*. Animals were anesthetized with sodium pentobarbital and killed by rapid decapitation, followed by complete bloodletting. Tissues and organs were immediately dissected, frozen in liquid nitrogen, and stored at –80°C until use.

RNA preparation and cDNA synthesis

Total RNA was extracted using ISOGEN (NIPPON GENE, Tokyo, Japan). The cDNAs used as templates for RT-PCR were synthesized from 3 μg of denatured total RNA using 5 μM oligo(dT) primer and 100 units of M-MLV Reverse Transcriptase (Promega, Madison, WI) in a 20 μl reaction volume with incubation at 42°C for 1.5 h. The cDNA used for rapid amplification of cDNA ends (RACE) was synthesized from 3 μg of total RNA using a SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions.

Molecular cloning of TR cDNAs by RT-PCR and RACE

RT-PCR was carried out to obtain partial TR cDNAs from skin cDNA using degenerate primers (Table 1). All of the following PCR amplifications were performed in 20 μl reaction volumes containing each primer at 1 μM, 0.25 unit of TaKaRa Ex Taq (TaKaRa, Shiga, Japan), each dNTP at 250 μM, and Ex Taq Buffer (TaKaRa). The PCR product was separated by electrophoresis, extracted using a QIAquick Gel Extraction Kit (QIAGEN K.K., Tokyo, Japan) and directly sequenced. After determination of the partial sequence, RACE was carried out to determine the complete sequence of TR cDNAs. PCR and nested PCR were performed with gene-specific primers (Table 1) in combination with Universal Primer A Mix (Clontech) or Nested Universal Primer A (Clontech). The amplified products were sequenced as described above. This procedure was repeated independently at least twice to avoid PCR amplification errors.

Comparison of the amino acid sequences of various TRs

CLUSTAL X software (version 1.81) (Thompson *et al.*, 1997) was used with default settings to align the deduced amino acid sequences of the TRs of the leopard gecko and other species.

Amino acid identities were calculated for the C domain, E/F domain, and entire ORF.

Molecular phylogenetic analysis

The nucleotide sequences of the entire ORFs of the TRs from the leopard gecko and from several species representing all other vertebrate classes were aligned using CLUSTAL X with default settings. The alignment of the nucleotide sequences was used to generate a phylogenetic tree, using the neighbor-joining method (Saitou and Nei, 1987). Bootstrap values were calculated with 1000 replications to estimate the robustness of internal nodes. The GenBank accession numbers of TRs used in the phylogenic analysis are as follows: *Homo sapiens* (human) TRα, **M24748**; *Homo sapiens* TRβ, **X04707**; *Mus musculus* (mouse) TRα, **MMCERBA1**; *Mus musculus* TRβ, **S62756**; *Gallus gallus* (chicken) TRα, **Y00987**; *Gal-*

Table 1. Oligonucleotide primers used for RACE, RT-PCR, and sequencing.

Name		Nucleotide sequence	Usage
TRα	SE01	GCCGCTCGAGGATCCCATTTCCGTG	Seqencing
	SE02	ACCCGNAAYCAGTGYCAGYTS	Degenerate PCR
	SE03	ATGCTGAAATCTCTTCAGCATCGG	Seqencing
	SE04	CCTCAGACCGCAGTGGGCTGATCTGC	3' -RACE
	SE05	CCGACCTGCGCATGATTGGGGCTTGC	Seqencing
	SE06	CCCCACCTCATCACCTCGGACACAAC	Seqencing
	AS01	TTSGGCCAGAAGTGVGGAAT	Degenerate PCR
	AS02	CTTTGTCCCCATCGGGCATGGAGG	Seqencing
	AS03	GCCGTCGGCTCTGGCCGATGCTGAAG	5' -RACE
	AS04	CCTCTTTGCGCCGCCTCTCTCGGTTC	5' -RACE
	AS05	TTTACGTTTCCCATCCGGCCACCGG	Seqencing
	AS06	GTGTCCCAACCCCTATCACCAACGC	Seqencing
TRβ	SE01	GGGTCACACTACTCCTGTCTCCCAG	Seqencing
	SE02	CVMGNAAYCARTGYCARGAA	Degenerate PCR
	SE03	AGAGCTGCAGAAGACAATTGGGATA	3' -RACE
	SE04	TGGACAAGCACCAATAGTAAATGCC	Seqencing
	SE05	TGGGGAGATGGCAGTGACAAGGGGCC	3' -RACE
	SE06	GCCCAACAGAACTCTTTCCCCCTTTG	Seqencing
	SE07	GTTCTTGGAAGTCTTTGAGGATTAA	Seqencing
	SE08	CAATGCGGGTACTTGTGACAATTGC	Seqencing
	AS01	RTCYTCRAASACYTCYARGA	Degenerate PCR
	AS02	TTKGGCCARAARTGYGYMAC	Degenerate PCR
	AS03	CTTTCCCGCCTTCTGGGGCATTTA	5' -RACE
	AS04	GTCTTCTTCTGCAGCTCTTCCCGACG	5' -RACE
	AS05	GTAACTGGGTATGTACCCTGACATGC	Seqencing
	AS06	GTATTCTCAACGTCAAACTTTTCCA	Seqencing

Abbreviations for degenerate nucleotides: K, G or T; M, A or C; R, A or G; S, C or T; V, A or G; Y, C or T. N represents all four nucleotides.

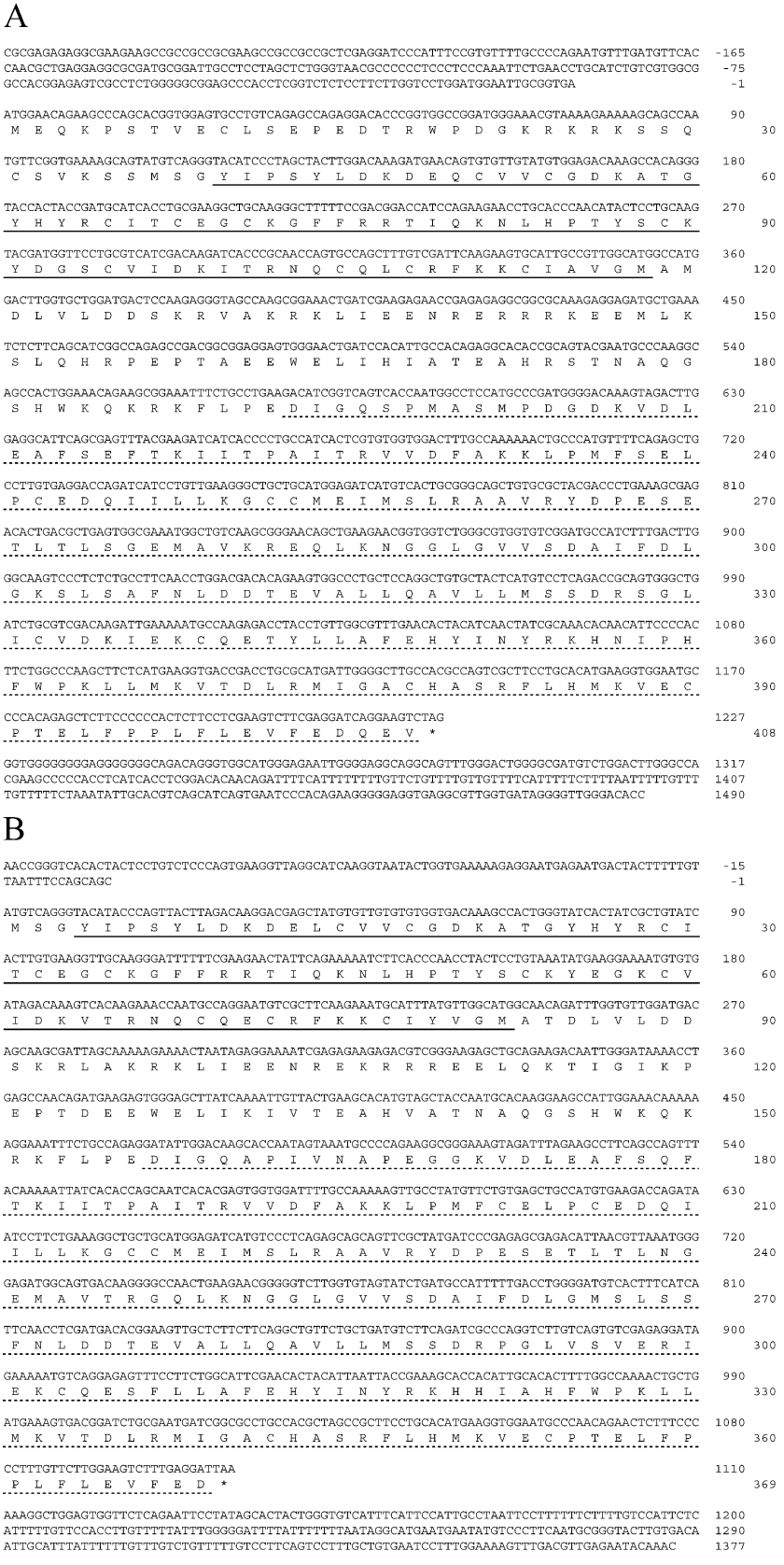

Fig. 1. Nucleotide and deduced amino acid sequence of the cDNA encoding **(A)** TRα and **(B)** TRβ of the leopard gecko. Nucleotides (upper row) are numbered from 5' to 3', beginning with the initiator codon (ATG) in the coding region. Amino acid residues (lower row) are numbered beginning with the first Met residue in the ORF. The C and E/F domains are indicated by solid and dashed underlining, respectively. The D domain is between these two domains.

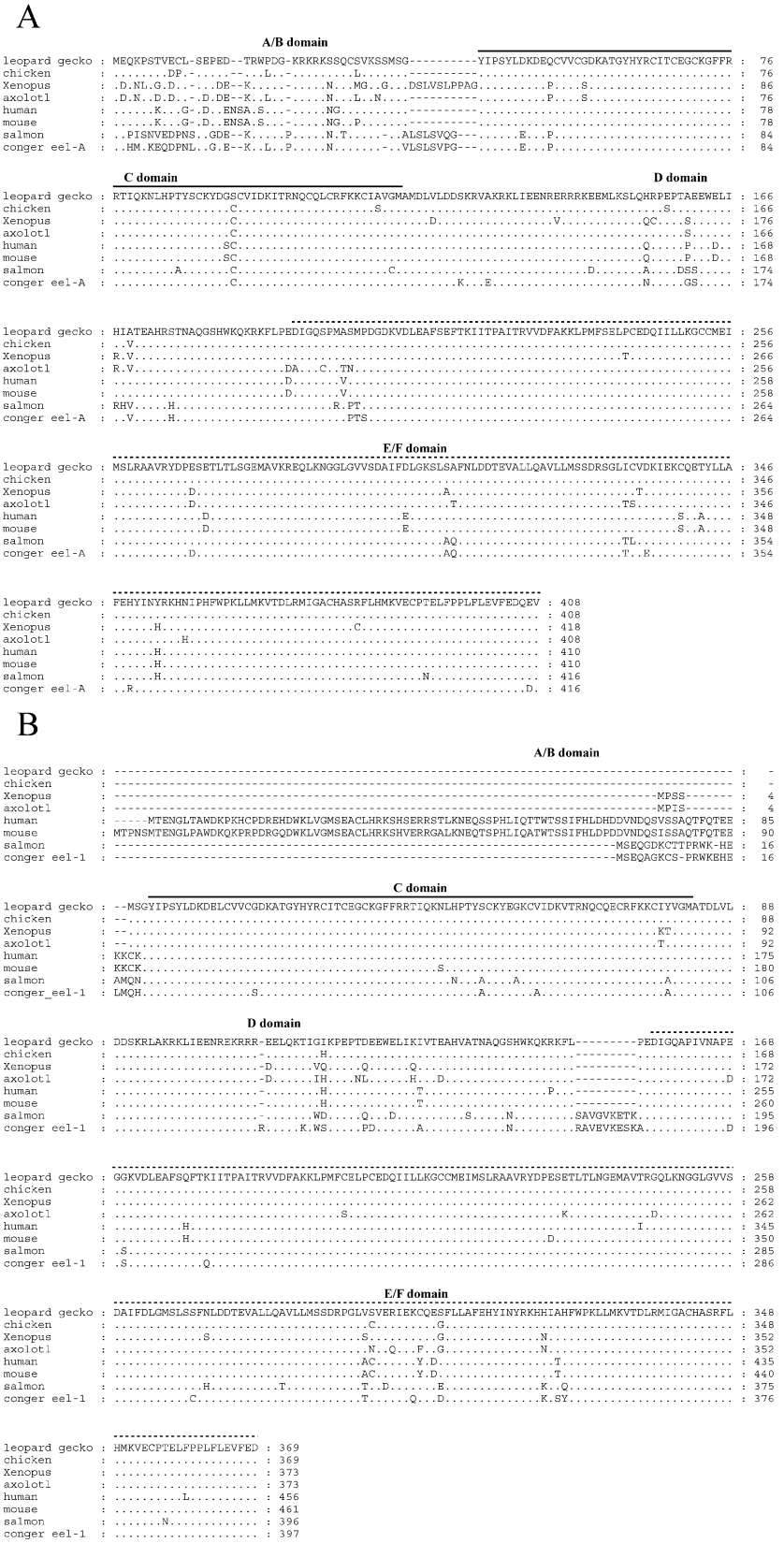

Fig. 2. Alignment of the predicted amino acid sequence of **(A)** TRα and **(B)** TRβ of the leopard gecko with homologs from other species. Dots indicate identity of amino acids with those of the TRs of the leopard gecko. Dashes indicate gaps inserted during alignment. The domains are indicated.

lus gallus TRβ, **X17504**; *Xenopus laevis* (African clawed frog) TRα, **M35344**; *Xenopus laevis* TRβ, **M35361**; *Ambystoma mexicanum* (axolotl) TRα, **AY174871**; *Ambystoma mexicanum* TRβ, **AY174872**; *Salmo salar* (salmon) TRα, **AF146775**; *Salmo salar* TRβ, **AF302251**; *Conger myriaster* (conger eel) TRαA, **AB183396**; *Conger myriaster* TRβ1, **AB183394**.

Expression analysis of TRs

To identify the target organs of THs, the spatial expression pattern of the TRs was examined by RT-PCR. Twenty-five nanograms of cDNA from the whole brain, heart, liver, small intestine, large intestine, testis, ovary, and thymus, and 5 ng from the pituitary, were amplified using primers specific for TRs. The primer sets used were TRαSE03 and TRαAS02 for TRα, and TRβSE03 and TRβAS03 for TRβ (Table 1). The PCR products were visualized by electrophoresis on a 1.2% TAE agarose gel and stained with ethidium bromide. Each DNA fragment was extracted from the gel and directly sequenced to confirm its identity.

Temporal expression analysis of TRs in skin

Total RNA was extracted from the skin of three animals within 24 hours before or after shedding. cDNA from the skin (7.5 ng) was amplified using the specific primers described above. The PCR conditions were as follows: 94°C for 3 min; 30 cycles of 94°C for 40 s, 64°C for 25 s, and 72°C for 30 s; and 72°C for 2 min. The PCR products were analyzed by electrophoresis on a 1.2% TAE agarose gel.

RESULTS

Molecular cloning of TR cDNAs from the leopard gecko

Full-length TR cDNAs were isolated from the skin of the leopard gecko by RT-PCR and RACE. TRα cDNA comprised 1,744 bp, which included a 5'-UTR of 254 bp, an ORF of 1,227 bp encoding 408 amino acid residues, and a 3'-UTR of 263 bp. TRβ cDNA comprised 1,481bp, including a 5'-UTR of 104 bp, an ORF of 1,110 bp encoding 369 amino acid residues, and a 3'-UTR of 267 bp. The domains are indicated in Fig. 1. Sequences of full-length cDNAs were deposited in GenBank (Accession Nos. **AB204861** and **AB204862**).

Comparison of the amino acid sequences of various TRs

Alignments of the predicted amino acid sequence of leopard gecko TRs (lgTRs) with those of other species are shown in Fig. 2. Across the entire ORF, the lgTRs showed very high identity (87–96% for TRα and 77–98% for TRβ) with their homologs from other species. When the specific domains were considered, stronger conservation was observed in the C domain (91–97% between lgTRα and its vertebrate homologs; 95–99% for TRβ) and in the E/F domain (94–95% between lgTRα and its vertebrate homologs; 92–99% for TRβ).

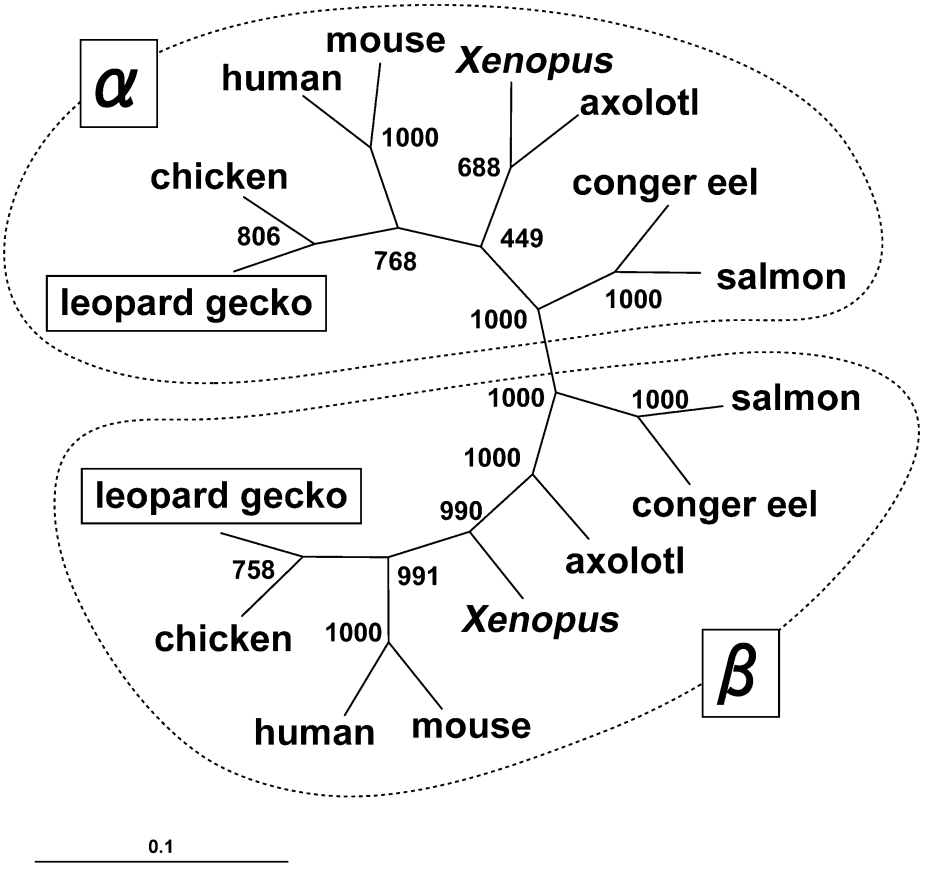

Fig. 3. Unrooted neighbor-joining phylogenetic tree of the TRs. The tree was constructed from the nucleotide sequences of the entire ORFs. Bootstrap values of 1000 resamplings are indicated for all nodes on the tree. The scale bar beneath the tree corresponds to the estimated evolutionary distance unit. Species names and GenBank accession numbers are given in Materials and Methods.

Molecular phylogenetic analysis

A phylogenetic tree of the TRs was constructed using the entire ORF nucleotide sequences of selected species representing all classes of vertebrate (Fig. 3). The TRs formed two groups, TRα and TRβ, in accordance with these two isoforms being derived from distinct genes. As expected, both the leopard gecko TRs clustered with their chicken homologs.

Expression analysis

As expected, a wide TR distribution was observed. RT-PCR products of the expected size were obtained from all tissues and organs examined (Fig. 4). The authenticity of the RT-PCR products was confirmed by direct sequencing. A control without RT was also used for 40 or 45 cycles of PCR, and no signal was detected (data not shown).

Fig. 4. Expression of the TR mRNAs in the leopard gecko. Five nanograms of cDNA from the pituitary, and 25 ng of cDNA from the whole brain, heart, liver, small intestine, large intestine, testis, ovary, and thymus, were subjected to PCR for TRs and β-actin of the leopard gecko.

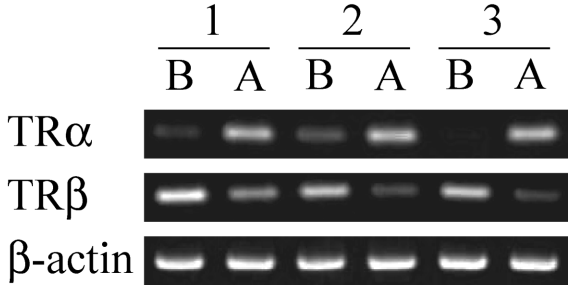

Fig. 5. Expression of TR mRNAs in the skin of the leopard gecko. The samples subjected to RT-PCR were taken from three animals before (indicated by "B") or after (indicated by "A") skin shedding. RT-PCR products beneath each horizontal bar were from mRNA taken from the skin of the same animal.

Temporal expression analysis in skin

The expression of the TRs in the skin was investigated by RT-PCR. TRα was expressed more strongly in the skin after shedding than before. In contrast, TRβ was expressed more strongly before shedding than after.

DISCUSSION

The THs are pleiotropic factors important for many functions in vertebrates. In reptiles, THs are suggested to affect tail regeneration, metabolic rate, metabolic enzyme activity, and shedding frequency. To augment the investigation of the molecular mechanism of THs in reptiles, we characterized their receptors at the molecular level.

In this study, we cloned two isoforms of TR from the leopard gecko, *Eublepharis macularius*. This is the first molecular identification of full-length TRs from reptiles. The deduced amino acid sequences of the cDNAs demonstrated the classic modular structure of members of the nuclear receptor superfamily, and exhibited high identity with their homologs in other species. The highest identities were shown with their chicken homologs (96 and 98%). There are eleven conserved Cys residues in the C domain of TRα. Although the seventh Cys in lgTRα, which does not contribute to the formation of a disulfide bond or zinc finger (Zhao *et al*., 1998), is substituted by Ser, other Cys residues are completely conserved in all TRαs. It is therefore conceivable that cloned lgTRα does not lose the ability to bind DNA by this substitution. In the future, ligand binding studies will be helpful. In the phylogenetic tree, TRs formed groups, TRα and TRβ. Both lgTRs clustered with their chicken counterparts, as expected.

mRNA expression of the lgTRs was detected in all the tissues and organs examined, indicating that thyroid hormones are pleiotropic factors important for many functions in the leopard gecko as well as other animals. In lizards, it has been reported that THs can regulate cardiac function (Venditti *et al*., 1996), enzyme activity in the liver (John-Alder, 1990), and testis activity (Cardone *et al*., 2000; Plowman and Lynn, 1973). It is therefore conceivable that TRs mediate such effects in these organs. In other species, the expression of TRs has been also demonstrated in various tissues, but significant isoform-specific functions are poorly understood. For instance, although the expression of TRs in the adult brain has been demonstrated in mammals, their specific roles have not yet been clarified (Schwartz *et al*., 1992). It is known that THs can regulate steroidogenesis; however, although the expression of TRs in the human ovary has been confirmed, their specific roles remain unknown (Zhang *et al*., 1997). There is less information for the testis, and the type of TR expressed there remains controversial (Maran, 2003).

THs are known to regulate the shedding frequency in Squamata. Intriguingly, the effect of thyroxine appears to be reversed between lizards and snakes. To obtain a better understanding of the potential role of THs in skin shedding, we analyzed the temporal expression of TRs in the skin. The expression of TRα was stronger after skin shedding than before, whereas the result was the opposite for TRβ. Although Chiu *et al.* (1967) have discussed the indirect effect of THs on shedding frequency, our results strongly suggest that THs can directly affect the skin, and that the two isoforms of TR play distinct roles in skin shedding.

The shedding cycle can be divided into two phases: resting and proliferation. As the skin is in the resting phase after shedding (Maderson and Licht, 1967), our result suggests that TRα plays a role in the resting phase, such as maintaining this condition so as not to enter the proliferation phase. Furthermore, we suspect that TRβ mediates the effect of THs in the proliferation phase, since TRβ was

strongly expressed in the skin before shedding. It is conceivable that the condition of the skin taken before shedding in this experiment was at around the last stage of the proliferation phase (Maderson and Licht, 1967). This is supported by an *in vitro* study demonstrating that the epidermis can differentiate by itself but cannot shed (Flexman *et al*., 1968). This indicates that the capacity for the complex changing pattern of cell differentiation is intrinsic to the epidermis, but that shedding is not. Extrinsic factor(s) is/are necessary for shedding, and THs may be one such factor. It has also been reported that THs have no effect on the skin in the proliferation phase, either directly or indirectly, as even thyroidectomized animals shed (Chiu *et al*., 1967). This discrepancy may be because it is always difficult to completely remove the thyroid surgically (Chiu *et al*., 1967). The interpretation of the physiological significance of up- or down-regulation of mRNA expression of TRs needs further study, such as *in situ* hybridization to analyze where and when during the shedding cycle TRs are expressed. The differential expression of TR isoforms has also been reported for other species, such as frogs during development (Sachs *et al*., 2000). Although both isoforms appear to be involved in regulating metamorphosis, their functional differences are not yet clear.

In this report, we characterized leopard gecko TRs and demonstrated the possibility of their direct involvement in skin shedding. These results will facilitate investigation of the physiological significance of THs and of the molecular mechanisms by which they regulate shedding frequency in Squamata.

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