

Molecular cloning and characterization of thyroid hormone receptors in teleost fish

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ABSTRACT

Thyroid hormones are pleiotropic factors important for many developmental and physiological functions in vertebrates. Their effects are mediated by two specific receptors (TR α and TR β) which are members of the nuclear hormone receptor superfamily. To clarify the function of these receptors, our laboratory has started a comparative study of their role in teleost fish. This type of approach has been hampered by the isolation of specific clones for each fish species studied. In this report, we describe an efficient reverse transcription/PCR procedure that allows the isolation of large fragments corresponding to TR α and TR β of a wide range of teleost fish. Phylogenetic analysis of these receptors

revealed a placement consistent with their origin, sequences from teleost fish being clearly monophyletic for both TR α and TR β . Interestingly, this approach allowed us to isolate (from tilapia and salmon) several new TR α or TR β isoforms resulting from alternative splicing. These isoforms correspond to expressed transcripts and thus may have an important physiological function. In addition, we isolated a cDNA encoding TR β in the Atlantic salmon (*Salmo salar*) encoding a functional thyroid hormone receptor which binds specific thyroid hormone response elements and regulates transcription in response to thyroid hormones.

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INTRODUCTION

Thyroid hormones (THs) play an important role in the growth, development and metabolism of vertebrates (Brent 1996). Abundant evidence demonstrates that most, if not all, of the biological effects of these hormones are mediated through the two thyroid hormone receptors, TR α and TR β (NR1A1 and NR1A2, respectively, in the nomenclature for nuclear receptors; Nuclear Receptors Nomenclature Committee 1999). TRs are members of a large superfamily of nuclear receptors which act as ligand-dependent transcription factors (reviewed in Gronemeyer & Laudet 1995). This family also includes vitamin D₃, steroid receptors and retinoid receptors. Nuclear receptors share conserved domains that are critical for their function: a short DNA-binding domain (DBD) encompassing two

zinc fingers, and a C-terminal hydrophobic ligand-binding domain (LBD) (Gronemeyer & Laudet 1995). They bind DNA specific sequences termed hormone response elements (HREs), which contain repeats of the consensus half-site sequence 5'-AGGTCA-3'. The arrangement and spacing of the half-sites within the HRE is thought to determine which receptors can bind and allow transcriptional regulation of the target genes (reviewed in Glass 1994). TH receptors, like many members of this superfamily, form heterodimers with RXR (NR2B), a receptor for 9-*cis* retinoic acid. This heterodimer binds the thyroid hormone response element (TRE). In most cases, these sequences consist of two half-sites arranged in direct repeats spaced by four nucleotides (DR4) (Umesono *et al.* 1991), but also include a palindromic element (HREpal) (Glass *et al.* 1988; for a review, see Desvergnès 1994). It

has been shown that, in the absence of hormone, the RXR–TR heterodimer binds to co-repressors and represses transcription (Chen & Evans 1995, Hörlein *et al.* 1995). In the presence of TH (mainly T₃, the tri-iodothyronine), the receptor undergoes conformational changes that induces the release of the co-repressors and the recruitment of co-activators (reviewed in McKenna *et al.* 1999). This results in a potent activation of target-gene transcription.

So far, two major types of TRs (α and β), encoded by separate genes, have been identified in mammals (Weinberger *et al.* 1986, Thompson *et al.* 1987), chickens (Sap *et al.* 1986), frogs (Yaoita *et al.* 1990, Schneider & Galton 1991) and fish (Yamano *et al.* 1994, Yamano & Inui 1995). Each of these genes can produce several isoforms by means of alternative splicing. For example, the human TR α gene can give rise to two different proteins, TR α 1 and TR α 2, that differ in the C-terminal region. Because it lacks most of the terminal region of the LBD containing the autonomous transcription-activating region, AF2-AD, the TR α 2 isoform is unable to bind T₃. Consequently, TR α 2 represses transcription, thus acting as a dominant negative regulator of TR α 1, which acts as a regular TH receptor (Koenig *et al.* 1989, Lazar *et al.* 1989, Katz & Lazar 1993). In the rat, the TR β gene can also produce two proteins, TR β 1 and TR β 2, that differ in the N-terminal region (Hodin *et al.* 1989). These two isoforms exhibit very different expression patterns: TR β 1 is expressed in many organs and shows complex regulation during embryogenesis, whereas TR β 2 is expressed only in the pituitary gland (Hodin *et al.* 1989, 1990). The *Xenopus* TRs also encode multiple isoforms that differ in the N-terminal region (Yaoita *et al.* 1990).

Although the function of TRs has been extensively scrutinized in mammals, for example using knock-out technology (Fraichard *et al.* 1997, Gauthier *et al.* 1999), as well as in birds and amphibians, in which THs control metamorphosis (Tata 1997), little is known about TR function at the molecular level in fish. It has been shown that THs are required for flatfish metamorphosis, and that TRs are up-regulated during this process (Inui & Miwa 1985, Miwa *et al.* 1988, Yamano & Miwa 1998). More recently, the expression pattern of a TR α has been described in zebrafish (Essner *et al.* 1997), and it has been suggested that this gene is important in embryogenesis for repressing retinoic acid signaling pathways (Essner *et al.* 1999).

In order to study the function of THs and their receptors in a wide range of teleost fish, we designed an efficient reverse transcription/PCR (RT-PCR) method that allows the isolation of large cDNA

fragments corresponding to TR α and TR β from any teleost fish. We also isolated several isoforms, from tilapia and salmon, encoding splice variants of TR α and TR β and we showed that these isoforms correspond to bona fide transcripts. In order to characterize one of these receptors in greater detail, we isolated a putative full-size cDNA encoding TR β in the Atlantic salmon. We demonstrate that this receptor binds to direct repeat as well as palindromic elements and activates transcription in response to T₃.

MATERIALS AND METHODS

Reverse transcription and polymerase chain reaction

Given the nearly ubiquitous expression pattern of the TRs in vertebrates (Forrest *et al.* 1990, Fraichard *et al.* 1997), we used total RNA extracted from various tissues (muscle, gills, liver etc.). RT-PCR reactions were performed with RNA from a wide variety of teleost fish that represent most of the diversity of this very large clade (Nelson 1984): an anguilliform, the eel (*Anguilla anguilla*); two salmonids, the rainbow trout (*Oncorhynchus mykiss*) and the Atlantic salmon (*Salmo salar*); a pleuronectiform, the turbot (*Scophthalmus maximus*); a tetraodontiform, the fugu (*Takifugu rubripes*); a cypriniform, the zebrafish (*Danio rerio*); and a perciform, the tilapia (*Oreochromis niloticus*).

RNA from pufferfish was a generous gift of John Wentworth. Other RNA was extracted from frozen tissues of the fish. Total RNA was isolated from the whole body or from different organs by using the guanidinium thiocyanate method followed by purification with phenol–chloroform (Chomczynski & Sacchi 1987).

Total RNA (5 μ g) was reverse-transcribed using random primers or specific primers and Moloney murine leukaemia virus (M-MLV) reverse transcriptase in 20 μ l reaction mixture, according to the manufacturer's instructions (M-MLV-RT kit; Gibco-BRL, Eragny, France). The resulting cDNA was amplified by a PCR in 100 μ l with 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂ (Perkin-Elmer, Warrington, UK), 0.25 mM of each dXTP, 2.5 U *Taq* Gold DNA polymerase (Perkin-Elmer) and 300 ng of each primer.

Degenerate primers (DEG series; Table 1) were designed using an alignment of published human, rat, mouse, chicken and *Xenopus* nucleotide sequences, according to the method described in Escriva *et al.* (1999). Additional specific primers (FF and SA series; Table 1) were developed on the basis of preliminary sequencing results. These

TABLE 1. Degenerated oligonucleotides used in touch-PCR, 5'-RACE and inverted PCR essays

Target	Scientific name	Primer name	Primer sequence	
Eel Tilapia Zebrafish Pufferfish	<i>Anguilla anguilla</i> <i>Oreochromis niloticus</i> <i>Danio rerio</i> <i>Takifugu rubripes</i>	TR α	DEG1 (5'1) DEG2 (5'-nest) DEG3 (3'1) DEG4 (3'-nest)	AC (T/C) TG (T/C) GAGGTTG (C/T) AAGGG (C/A) GIACIAT (C/T) CA (G/A) AA (G/A) AACCT ATGTGIAG (G/A) AAGC (G/A) (G/A) CTGGC AG (C/G) AG (C/T) TT (G/C) GGCCAGAAGTG
		TR β	DEG5 (5'1) DEG6 (5'-nest) DEG7 (3'1) DEG8 (3'-nest)	ACITG (T/C) GA (G/A) GGITG (C/T) AAGGG (A/T) (C/G) (T/C) TA (T/C) (T/G) C (C/T) TG (C/T) AA (A/G) TA (T/C) GA GG (G/A) CACTCCAC (T/C) TTCATGTG AG (G/A) TCIGT (C/G) AC (C/T) TTCAT (C/T)
		TR α	FF1 (5'1) FF2 (5'-nest) FF3 (3'1) FF4 (3'-nest)	GGCTGCAAGGGTTTCTTCGG ATCCAGAAGAACCTCCACCC CCGATCATGGCAGGTCTGT GCAGCTTGGGCCAGAAAGTGG
		TR β	FF1 (5'1) FF6 (5'-nest) FF7 (3'1) FF8 (3'-nest)	GAAGGTTGTAAGGGTTTCTT AAGAACTCTGAACCCGACATA CTCGAAGACCTCCAGGAAGA GCTCGGTGGACACTCCACT
Salmon Trout	<i>Salmo salar</i> <i>Oncorhynchus mykiss</i>	TR α	See above DEG1 (5'1) DEG2 (5'-nest) SA1 (3'1) SA2 (3'-nest)	See above CTCCGTGTTCATCCAGGTTGAA GGCTCTTGGCCAGGTCGAAG
		TR β	DEG5 (5'1) DEG6 (5'-nest) SA3 (3'1) SA4 (3'-nest)	See above TCTGATACCACACCCAGGCC CCGTTCTTCAGCTGGCCGGG
Salmon Trout	<i>Salmo salar</i> <i>Oncorhynchus mykiss</i>	TR β	SA5 (5'-RACE) SA6 (5'-RACE) SA7 (5'-RACE)	CGAGCCGTTGCTGTCTGTCC TCTGTGCCATGCCGACAGC ACTTCTTGAAGCGCAATTC
		TR β	SA8 (inverted PCR) SA9 (inverted PCR nest) SA10 (inverted PCR) SA11 (inverted PCR nest)	TGATGTAATGTTCAAAGGCCAGAAGGAACT AAGAACTCCTCTGTCTGACAGCGCTCAATACGG CAAAACACAGGTGGCGCAATTTGGCCCAA ACCTGCGGATGATCGGGGCTGCCACGCTC
Salmon	<i>Salmo salar</i>	TR β	SA12 (prolongation 5'1) DEG11 (prolongation 3'1)	TTTAGGGGCGCGCTTCGGT GGG (A/G) CA (C/T) TCIA (A/C) (T/C) TTCATAT
		TR β	DEG9 (5'1) DEG10 (3'1)	TG (C/T) AA (A/G) TA (C/T) GA (A/G) GGIAA (A/G) TG TI (A/G) TG (C/T) TTIC (G/T) G (A/T) A (A/G) TT (A/G) AT

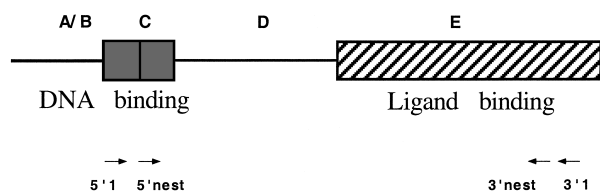


FIGURE 1. Experimental strategy for RT-PCR with degenerate primers. The degenerate oligonucleotides were chosen in the most conserved regions of the TH receptors, i.e. the DBD (C-domain) for the 5' primers and in the LBD (E-domain) for the 3' primers.

primers were located in the regions coding for the two conserved domains, allowing the amplification of large RT-PCR fragments (see Fig. 1). Most of these primers are degenerate and were used in a 'touch-down' PCR assay (Don *et al.* 1991) during which the hybridization temperature is reduced by 5 °C every five cycles to allow hybridization of at least one of the primers of the degenerated mix; denaturation and elongation temperatures remain unchanged. Thus, the complete cycle was as follows: 94 °C, 1 min; hybridization at 55–37 °C, 1 min; 72 °C, 1 min; 40 cycles. To avoid contamination, all of the RT-PCR reactions as well as RNA extraction were conducted in a special nucleic-acid-free room under positive air pressure and using specific reagents (Safi *et al.* 1997, Hänni *et al.* 1994). For each pair of primers used, we prepared a control reaction (without DNA) to check that our PCR reactions were not contaminated (in order to avoid

spurious amplification of TRs from mammals routinely used in our laboratory).

To study the expression of the various isoforms, we designed the specific primers described in Table 2 and Fig 2A and B. Other primers (TNA1 and -2, TNB1 and -2, SSA1 and -2; Table 2) were used as positive controls for PCRs. In these cases, we used a standard PCR protocol consisting of 40 cycles of the following: denaturation at 94 °C, 1 min; hybridization at 55 °C, 1 min; elongation at 72 °C, 30 s. PCR products were electrophoresed on agarose gels and stained with ethidium bromide.

Cloning and analysis of cDNA

PCR products were cloned into the PCR II vector (Invitrogen, Groningen, The Netherlands). Each plasmid was sequenced with a 373A ABI automated DNA sequencer with the Prism Ready Reaction Dyedeoxy Terminator cycle sequencing kit (Perkin-Elmer). For each amplified TR, two independent clones were fully sequenced and the sequences compared in order to avoid mutations generated by the TAQ Gold polymerase. In case of mismatches, a third clone was sequenced for confirmation of the correct sequence. Each final sequence was then compared with reference sequences, using the FASTA program (the 10–3 option in the CITI Bisance/Infobiogen network: <http://www.infobiogen.fr>).

The full-size salmon TR β cDNA was obtained using 5'-RACE (rapid amplification of cDNA ends) analysis (Frohman *et al.* 1988), an additional RT-PCR with degenerated probes, as well as

TABLE 2. Oligonucleotides used in isoform detection

Target	Primer name	Primer sequence	Size of PCR product (pb)
Tilapia TR α	TNA1 (5')	GCTGCATCATCGACAAGATC	654
	TNA2 (3')	GATCTGAGCTCATGAGAAGC	
Tilapia TR β	TNB1 (5')	AATGTGTTATTGACAAAGTG	654
	TNB2 (3')	GATCGGATGAAAGCAGGATA	
Tilapia TR α , small isoform	TNA3 (5')	AGAAGCGCAAATTCCTGCTG	381
	TNA2 (3')	See above	
Tilapia TR α , large isoform	TNA4 (5')	AGAAGCGCAAATTCCTGCCA	234
	TNA2 (3')	See above	
Salmon TR α	SSA1 (5')	GCATGGCCATGGACCTCGTT	290
	SSA2 (3')	CCGTCCGACGTGGGGACCAT	
Salmon TR β , small isoform	SSB1 (5')	TCACTGTCCGGCATGGCAACA	246
	SSB2 (3')	TGACCAATGTCTCTCAGGCAA	
Salmon TR β , large isoform	SSB1 (5')	See above	260
	SSB3 (3')	CGGGCTTGGTTTCCTTACC	

pb, Pairs of bases.

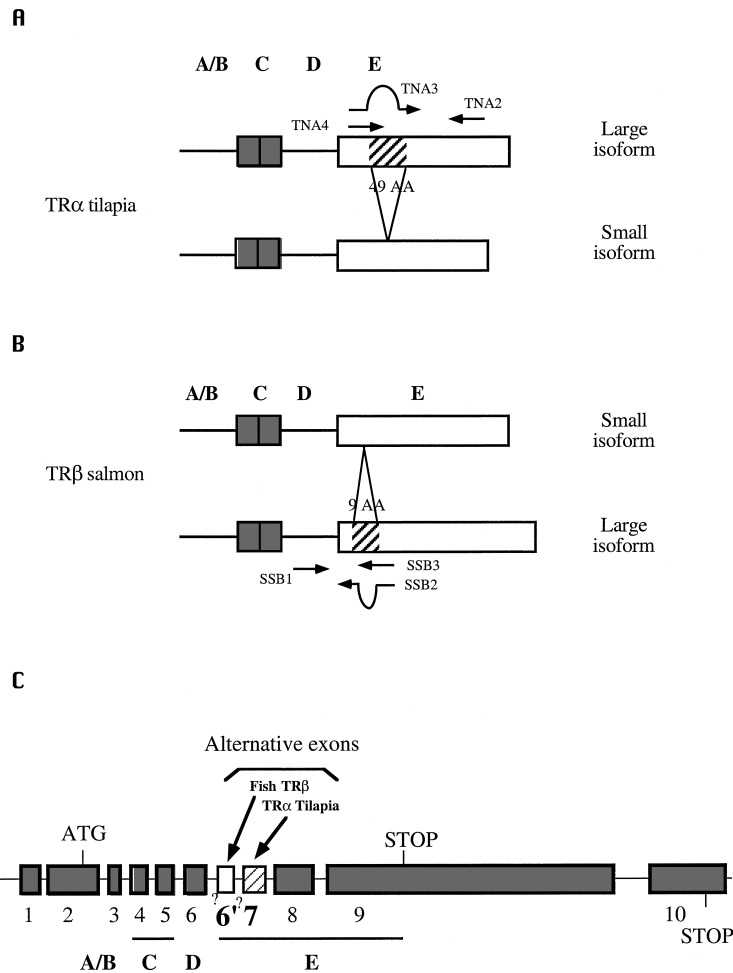


FIGURE 2. (A) Structure of the tilapia TR α isoform. The deletion of 49 amino acids ('49 AA') concerns the central part of the LBD. (B) Putative structure of the salmon TR β isoform. The nine-amino-acid ('9 AA') insertion corresponds to a region located at the beginning of the LBD, between H2 and H3. For panel (A) and (B), the primers used to test for the expression of isoforms are indicated by arrows. (C) Genomic organization of the human TR α gene (Laudet *et al.* 1991) in which the corresponding teleost isoforms were located. The tilapia TR α isoform corresponds to a deletion of an exon homologous to exon 7 in the human TR α gene. We presume that the nine-amino-acid insertion corresponds to an alternative exon 6 of 27 bp, although we cannot exclude the possibility that it corresponds to the use of an alternative splice donor or splice acceptor site from a larger exon 6, or exon 7. The functional domains are indicated above the genomic structure. Exons are represented here in proportion to their size in bp, whereas intron lengths shown here are arbitrary.

inverted PCR reactions using genomic DNA. To clone the 5'-region of the cDNA, a 5'-RACE reaction was performed using the 5'/3' RACE kit from Boehringer (Meylan, France), according to the manufacturer's instructions. The oligonucleotides

(SA5, SA6, SA7) used in this experiment are shown in Fig. 3 and Table 1. For the 3'-end, we first performed another RT-PCR (with SA12 and DEG11 probes) and the resulting fragments allowed us to design other specific oligonucleotides for

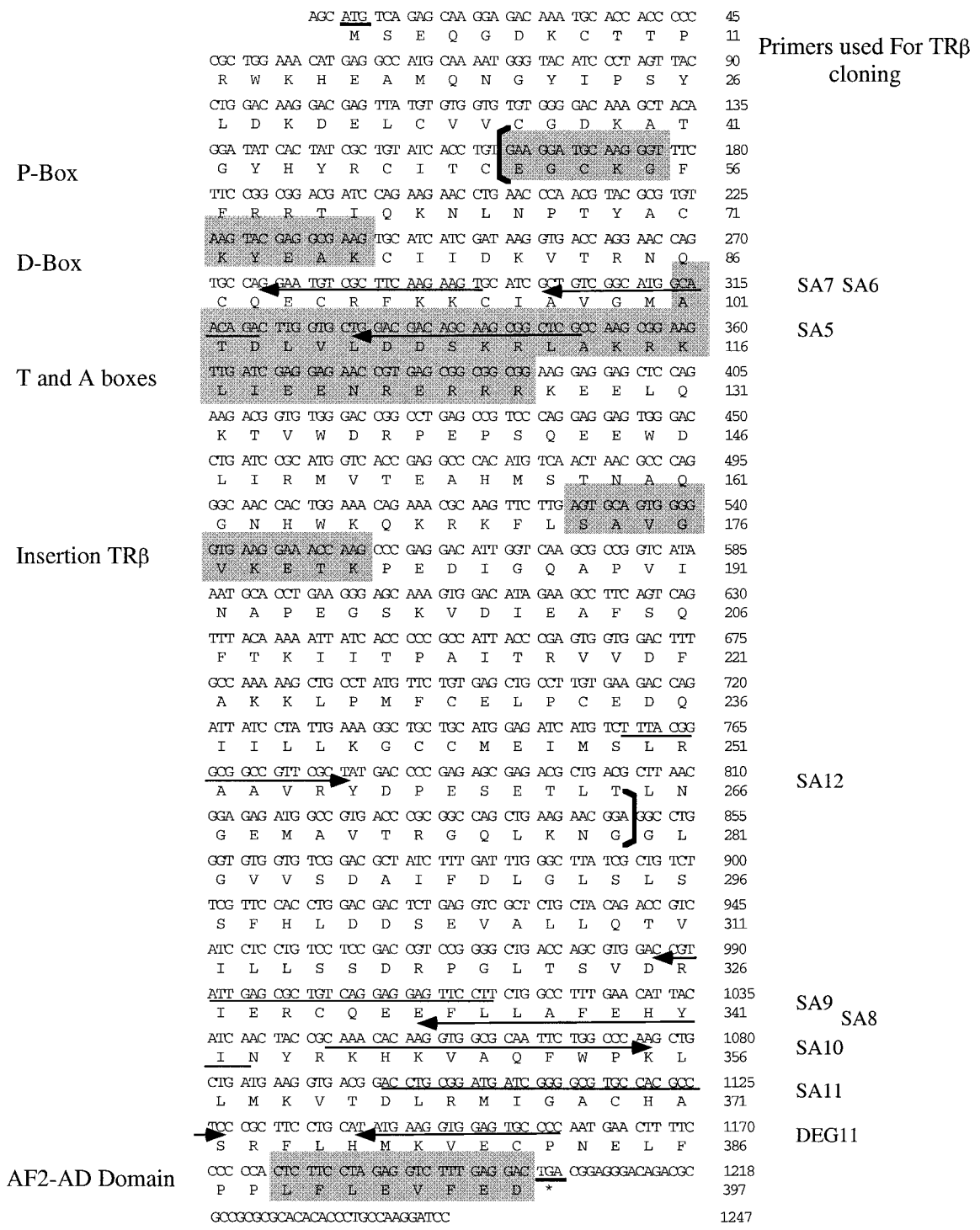


FIGURE 3. Sequence of the *Salmo* TRβ. The limits of the first RT-PCR product are indicated. The region obtained by 5'-RACE corresponds to positions 1–165 and those obtained by new RT-PCR in 3' correspond to positions 848–1158. Inverted PCR allowed us to obtain 3' sequence up to the stop codon. Start and stop codons are underlined and the teleost-specific alternative nine-amino-acid insertion is boxed. Note that we are not sure of having the true first initiation codon of the cDNA, since the reading frame upstream of this methionine is open. Functional domains (DBD, T/A boxes implicated in DNA binding and dimerization; LBD, AF2-AD core region) are indicated.

inverted PCRs (Fig. 3, Table 1). This technique was performed as described in Ochman *et al.* (1988) and Triglia *et al.* (1988).

Phylogenetic analysis

Amino acid sequences of the various proteins were aligned using SEAVIEW, which allows manual alignment (Galtier *et al.* 1996). The GenBank accession numbers of the sequences used are as follows: *Homo sapiens* TR α (M24748), *Rattus rattus* TR α (M18028), *Mus musculus* TR α (X51983), *Gallus gallus* TR α (Y00987), *Cairina moschata* TR α (Z50080) v-erbA (K02006), *Danio rerio* TR α (U54796), *Xenopus laevis* TR α A (M35343), *Xenopus laevis* TR α B (M35344), *Rana castebeina* TR α (L06064), *Paralichthys olivaceus* TR α A (D16461), *Paralichthys olivaceus* TR α B (D16462), *Hippoglossus hippoglossus* TR α (AF143296), *S. salar* TR α B (AF146775) *H. sapiens* TR β (X04707), *R. rattus* TR β (J03933), *M. musculus* TR β (S62756), *G. gallus* TR β (X17504), *C. moschata* TR β (Z49151), *X. laevis* TR β A (M35360), *X. laevis* TR β B (M35361), *R. castebeina* TR β (L27344), *P. olivaceus* TR β (D45245), *S. salar* TR α (AF302250), *Oncorhynchus mykiss* TR α (AF302245), *S. maximus* TR α (AF302253), *Oreochromis niloticus* TR α 1 (large isoform; AF302248), *O. niloticus* TR α 2 (small isoform; AF302249), *T. rubripes* TR α (AF302243), *A. anguilla* TR α (AF302240), *S. salar* TR β 1 (large isoform; AF302251), *S. salar* TR β 2 (small isoform; AF302252), *O. niloticus* TR β 2 (small isoform; AF302247), *O. mykiss* TR β (AF302246), *D. rerio* TR β (AF302242), *S. maximus* TR β (AF302254), *O. niloticus* TR β (AF302247), *T. rubripes* TR β (AF302244), *A. anguilla* TR β (AF302241).

Because of the variability in the sequences, we have included only the DNA-binding domain, also called the C-domain, the D-domain and the LBD (also called the E-domain). A global gap-removal option was used and thus only those regions present in all aligned sequences were used to construct the trees. The data set was subjected to distance analysis with the neighbor-joining method (the NJ option of PHYLO_WIN; Galtier *et al.* 1996). For this analysis, 1000 bootstrap replicates were carried out.

Electrophoretic mobility-shift assays

Full-length *Salmo* TR β and *Xenopus* RXR α proteins were translated *in vitro* using the TNT kit (Promega, Madison, WI, USA). A DR4 probe from the *Xenopus* TR β promoter encompassing the transcription start site (positions -12 to 18; Machuca *et al.* 1995) was labeled with T4 polynucleotide kinase in the presence of

[γ -³²P]dATP. Binding reactions were performed using a radiolabeled DNA probe (1 ng) and wheat-germ lysate in 10% glycerol, 10 mM Hepes, 30 mM KCl, 4 mM spermidine, 0.1 mM EDTA, 0.25 mM dithiothreitol, 1 mM Na₂PO₄, and poly (dI-dC) (1.5 μ g) for 10 min. Unlabeled competitor oligonucleotides were included at the indicated molar excess in the binding reactions. Samples were loaded on a 5% non-denaturing polyacrylamide gel and allowed to migrate for 2 h at 180 V.

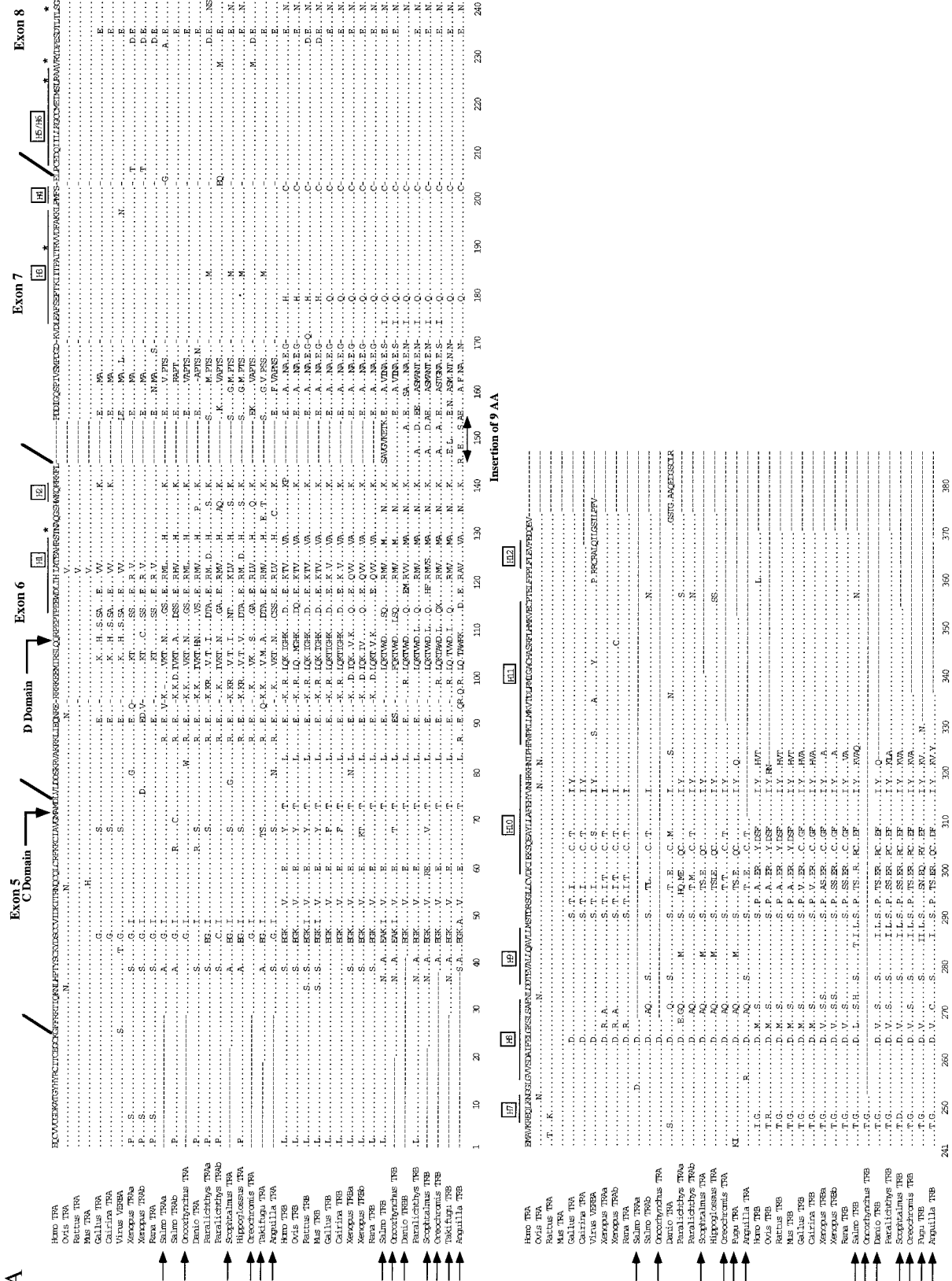
Transient transfection assays

The salmon TR β cDNA was subcloned into BamHI/XhoI sites from a modified pSG5 vector. Ros 17.2/8 (rat osteosarcoma) cells were plated in Dulbecco's modified Eagle's medium (Bio Media, Bousquet, France) supplemented with 10% calf serum (FCS), at a density of 10⁵ cells per dish. Transient transfections were performed using ExGen (Euromedex, Souffelweiersheim, France) with 0.1 μ g of the reporter plasmid (DR4-pGL2; Promega) together with 0.1 μ g of the TR β -producing plasmid and 0.8 μ g pSG5 as a carrier. In control transfections, 0.1 μ g of the reporter plasmid and 0.9 μ g pSG5 were used. Cells were washed after 6 h and fresh medium was added together with different concentrations of T3 (from 10⁻¹¹ to 10⁻⁶ M). Cells were lysed 48 h after transfection, and luciferase activity was measured using standard methods. Transfections were performed in triplicate.

RESULTS

RT-PCR isolation of TRs from a wide variety of teleost fish

In order to isolate specific probes for TR α or TR β from a wide variety of fish, we designed a series of degenerated PCR primers that can be used after reverse transcription. For each fish RT-PCR, fragments were obtained at the expected size for TR α or TR β (data not shown). From each PCR fragment, two clones were selected for sequencing. The resulting sequences were aligned with known TRs from vertebrates to check their authenticity (Fig. 4A). Surprisingly, the salmon TR α we cloned is different from that which is already present in GenBank (S A Rogers, G E Sweeney & T Wigham, unpublished observations; accession number AF146775). Although our RT-PCR clone for salmon TR α (called TR α A in Fig. 4) is closely related to the TR α clone from rainbow trout, the previously known TR α (called TR α B) clearly appears to be different. This has been confirmed by phylogenetic analysis (see below and Fig. 4B).



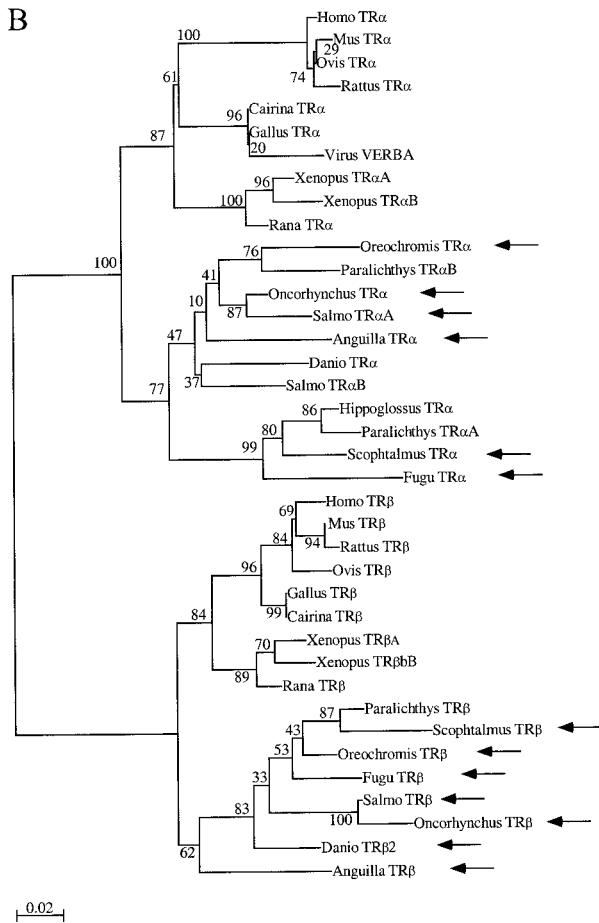


FIGURE 4. (A) Alignment of the amino acid sequences from the TRs isolated in this study, compared with known TRs from the literature. Only sequences from the conserved C, D and E domains are shown. Dots indicate amino acids identical to the first line, which correspond to the human TR α . Dashes indicate gaps inserted to enhance sequence similarity. The limits of the C and D domains are indicated. The positions of the helices found in the three-dimensional structure of the DBD and LBD of TR α are indicated at the top of the figure. The amino acids known to be directly in contact with the ligand in the three-dimensional structure are indicated by asterisks. The teleost-specific nine-amino-acid insertion in TR β is indicated by \longleftrightarrow and the position of an intron is indicated by a slash in the first line. Positions are numbered below the alignment, taking, as number 1, the beginning of the C-domain, two amino acids upstream of the first conserved cysteine. (B) Phylogenetic analysis of TR sequences aligned in (A). A global gap-removal option was used. The arrows indicate the sequences obtained in this work. The bootstrap values of each branch are indicated. This tree has been rooted with several nuclear receptors, including retinoic acid receptors (RARs), which are the nuclear receptors closest to the TRs.

Teleost TR sequences exhibit strong sequence identities with known TR α and TR β . Overall, fish TR α exhibits 86% and TR β 85% amino acid identity with their respective human orthologs. As expected, the region of the DNA-binding domain that we have isolated using our RT-PCR strategy, which roughly corresponds to the second zinc finger, is strongly conserved. Note that in the full-length salmon TR β clone that we have isolated (see below), the P-box is perfectly conserved, suggesting that the fish TRs are likely to exhibit the same DNA-binding properties as their homologs in other vertebrates. The T and A boxes which are immediately adjacent to the C-domain, and which play an important part in DNA binding and dimerization, forming a long α -helix, are strongly conserved. Two positions are diagnostic for TR β versus TR α in this region (M72 and V82 for α , T72 and L82 for β). Note that one position (K86) is specifically changed in R for fish TR α . This observation supports the authenticity of our RT-PCR fragments. Although it contains more mutations than the other domains, the D-domain is clearly conserved among TR α or TR β sequences. The LBD is strongly conserved. For example, helices deduced from the crystal structure of the rat TR α LBD are well conserved for all sequences examined (Wagner *et al.* 1995). This is especially true for H3, H5, H6 and the two C-terminal helices, H11 and H12, that we have sequenced in the *Salmo* TR β clone. In the available sequences, the AF2-AD region at the extreme C-terminus is strongly conserved. Moreover, the amino acids known to interact directly with the TH are conserved, with one exception: position 240 (which is a Ser residue in TR α from all known vertebrates) is substituted by an Asn residue in the TR α sequences from flatfish (*Paralichthys*, *Scophthalmus* and *Hippoglossus*), except for the duplicated TR α B gene of *Paralichthys*. In the LBD, five positions are strictly specific to teleost TR α , whereas three are specific for teleost TR β . Overall, TR α appears to be slightly more variable than TR β . This is reminiscent of the situation observed in other species, suggesting that TR β sequences are more constrained than TR α sequences (Laudet 1997).

Another interesting feature is that all known TRs β from teleosts contain an insertion of nine amino acids (as indicated by \longleftrightarrow in Fig 4A) between helices H2 and H3. This insertion had already been found in the TR β of flounder, as previously described (Yamano & Inui 1995), and has never been found in mammals or amphibians. It is located just after a region containing a short β -strand that forms the edge of the mixed sheet parallel to S4 in the three-dimensional structure of TR α .

In order to prove that our sequences were not PCR contamination products but effectively from teleost fish, we constructed a phylogenetic tree using the distance matrix method (Fig. 4B). In the tree, the TR α and TR β sequences are clearly separated in accordance with the fact that these two genes are the products of an ancient gene duplication event that arose early on during vertebrate evolution (Laudet *et al.* 1992, Laudet 1997, O Marchand & V Laudet, unpublished observations). For both TR α and TR β , the teleost sequences are grouped together (albeit with relatively modest bootstrap support of 77 and 62% respectively), whereas mammals, birds and amphibians (i.e. tetrapods) are arranged in another well-supported monophyletic group (87 and 84%). The topology of the TR β tree is in accordance with the known phylogeny of teleost fish, despite low bootstrap values (33–83%). For instance, we found the two flatfish (*Paralichthys* and *Scophthalmus*) grouped together with the tilapia (*Oreochromis*). This observation is consistent with the fact that flatfish and perciforms are both members of the very large and diverse percomorphs (Rosen 1973, Lauder & Liem 1983, Johnson & Patterson 1993). This group is then joined by the fugu and the two salmonids. Finally, the zebrafish clusters with these fish and forms a strongly supported clade (83% bootstrap) that corresponds to the Euteleostei (Greenwood *et al.* 1966, Patterson & Rosen 1977). The anguilliform (the eel), which is an early offshoot of teleost fish, is the 'sister' group of this clade (Nelson 1984). The situation is quite different for the TR α tree. There are two clusters in the teleost TR α tree. The first cluster is strongly supported (99% bootstrap) and joins the sequences of three flatfish (*Paralichthys*, *Scophthalmus* and *Hippoglossus*) and the sequence from *Fugu*. Interestingly, this cluster excludes the fourth sequence of flatfish (*Paralichthys* TR α B) which corresponds to a second TR α sequence found in *Paralichthys* (Yamano *et al.* 1994), as well as the tilapia sequence, which would have to be grouped with the flatfish in order to conform with teleost phylogeny, as for TR β . The second cluster groups all the other TR α sequences. The relationships between these sequences are unclear because of very low bootstrap values, and only two groups are discernible: *Paralichthys* TR α B and *Oreochromis* TR α (two percomorphs) cluster together, with 76% bootstrap values; and *Oncorhynchus* TR α and *Salmo* TR α A, two salmonids, cluster together, with 87% bootstrap values. Notably, the second TR α sequence from salmon (*Salmo* TR α B) does not cluster with *Salmo* TR α A. The reason why the phylogenetic relationships of the TR α sequences are scrambled, unlike

the TR β sequences, is presently unclear. The explanation might be linked to the observation that some TR α genes were duplicated (at least in *Salmo* and *Paralichthys*), whereas this has never been observed for TR β (see the Discussion).

Presence of alternative isoforms in tilapia and salmon

In some cases, we have obtained shorter sequences for both TR α and TR β . For tilapia, we observed a deletion of 147 nucleotides TR α (not shown). Strikingly, the boundaries of this deletion correspond precisely to the boundaries of human exon 7, suggesting that this exon may be skipped in some mRNA transcripts (see Fig. 4A). In such cases, the resulting protein will contain an in-frame deletion of 49 amino acids (Fig. 2A). Such proteins will not contain the region coding for helices H3 and H4, and are unlikely to bind T3, since this region encompasses some of the residues that are directly in contact with the ligand. To check that this isoform does not result from an RT-PCR artefact, gill RNA from tilapia was analysed by PCR with specific primers for either the large or the small isoform (Figs 2A and 5A; Table 2). TR α - and TR β -specific primers were used as positive controls. Interestingly, we observed a band of the expected size for both the large and the small isoform, although the small isoform appears to be present in smaller amounts than the large one. This suggests that this isoform is expressed and should correspond to a bona fide transcript.

For TR β , we noticed in salmon that the nine-amino-acid teleost-specific insertion was absent, suggesting that it may correspond to an alternative exon (Fig. 2B). We used RT-PCR to check for the expression of this isoform in gill RNA and found that both isoforms, with or without the 27 bp insertion, are expressed (Fig. 5B). Since this insertion is an alternative one in the flounder, we conclude that it is likely that the nine-amino-acid insertion present in teleost TR β corresponds to an alternative exon that is able to generate two different proteins.

Characterization of the salmon TR β

Using a 5'-RACE strategy (from gill mRNA) and inverted PCR, we constructed a cDNA encoding the large isoform of salmon TR β . This cDNA is 1247 bp long and contains an open reading frame predicted to encode a 397-amino-acid protein (Fig. 3). Since the reading frame is open before the first methionine, we are not sure whether this clone is really full size. Nevertheless, its comparison with

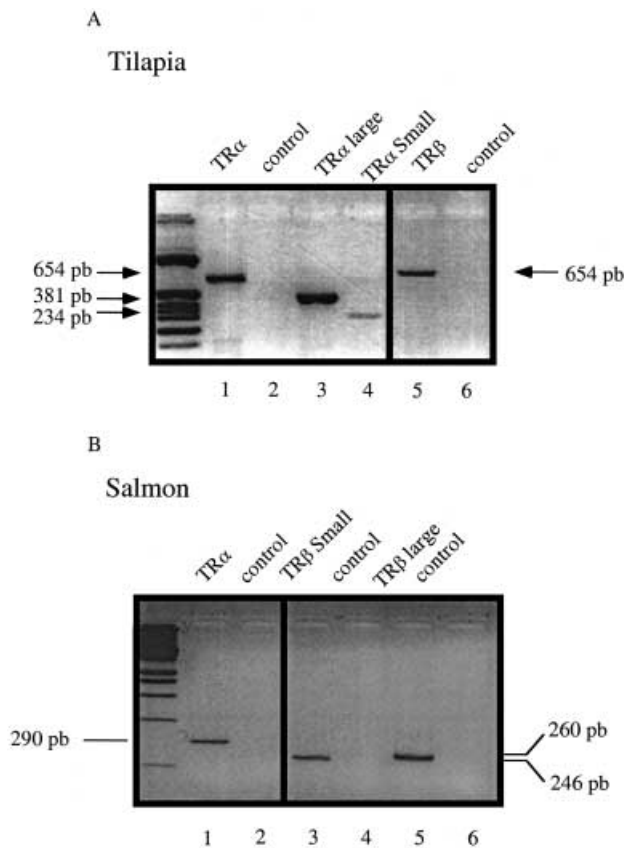


FIGURE 5. Expression of the TR isoforms in teleost fish studied by RT-PCR with the primers indicated in Table 2 and Fig. 3A and B. (A) Expression of the TR α isoform in tilapia gills. (B) Expression of the TR β isoform in salmon gills. In each case, the sizes of the expected PCR products are indicated. For each species, the expression of TR α and TR β (without taking the isoforms into account) was studied as a positive control using specific primers located in the C and E domains. Control lanes correspond to RT-PCR reactions done without adding RNA to ensure that there was no contamination of the reagents. pb, Pairs of bases.

TR β from other fish (*Paralichthys*; Yamano & Inui 1995) and amphibians leads us to believe that the indicated ATG is the correct one. Since this cDNA encodes a protein containing the complete, main, functional domains of TR β , we decided to characterize it in greater detail.

We used gel-shift assays to test whether salmon TR β binds to DNA. We used three types of TRE: a consensus DR4 sequence, the xDR4 found in the promoter of the *Xenopus* TR β gene (Machuca *et al.* 1995); a palindromic element (HREpal; Glass *et al.* 1988), and a complex TRE found in the regulatory region of the rat myelin basic protein (MBP) gene

(Jeannin *et al.* 1998; Fig. 6A). This element is interesting since it is a palindrome formed by two hexamers spaced by 6 bp, which confers differential binding ability for rat TR α 1 or TR β . As shown in Fig. 6B, the salmon TR β is able to bind xDR4 (lane 4); this binding is specific as it can be competed out by the same probe (lanes 5–7) but not by an unrelated probe (lane 19). It can also bind a perfect consensus DR4 (lanes 8–10) as well as HREpal (lanes 11–13). It also binds the TRE found in the MBP promoter, but with much less efficiency than the two other elements, since with a higher concentration of probe (100 times more) we can detect only weak binding (lane 16).

We then examined the transactivating properties of salmon TR β by using transient transfection assays. These assays were performed in osteosarcoma Ros 17.2/8 cells, since these cells contain a high level of RXR sufficient to provide a strong heterodimerization partner for the transfected TR. In addition, these cells contain low levels of TR, thus avoiding interference between the endogenous and transfected receptors (J M Vanacker, personal communication). The results show that this large isoform is able to activate transcription of the luciferase gene by binding to the xDR4 probe in a T3-dependent manner. Interestingly, without hormone it can repress the basal level of expression of luciferase (compare lane 3 with lane 1). A dose/response study shows that the salmon TR β is sensitive to 10^{-9} M T3, and that its transactivation ability increases up to 10^{-6} M (Fig. 6C).

DISCUSSION

Cloning and phylogenetic analysis of teleost TRs

In this study, we have set up an RT-PCR strategy that allows the isolation of large probes corresponding to the TH receptors α and β in a wide variety of teleost fish. The choice of species used, i.e. basal teleosts such as the eel (a non-euteleost fish) as well as members of a derived order of euteleostei such as flatfish (percomorphs), makes us confident that, using the same primers, it will be possible to isolate the TRs of any teleost. In fact, we have been able, with these primers, to isolate TRs in other vertebrates such as the shark, the lamprey or the hagfish, as well as in several amphibians (O Marchand, R Safi, V Laudet, L Manzon & J Youson, in preparation). Given the importance of THs for the control of numerous physiological processes and in developmental events such as metamorphosis, this method could have important applications.

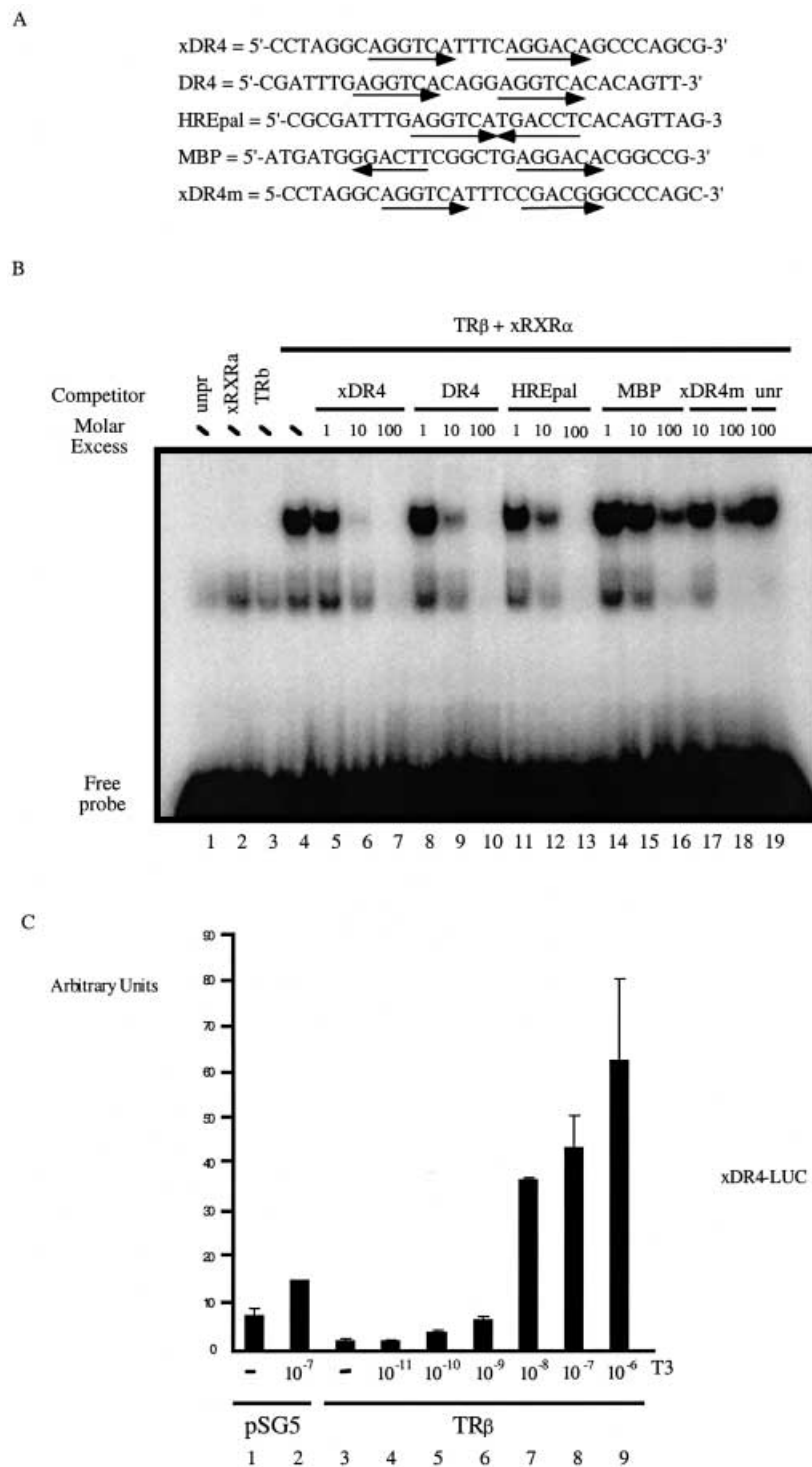


FIGURE 6. (A) Sequence of the binding sites used in the gel-shift experiments. The consensus core element is indicated by arrows. (B) Gel-shift experiments. The labeled probe used is the xDR4 in competition with the others at the indicated molar excess. 'Unpr', unprogrammed probe (the negative control); 'unr', unrelated probe; backward slash, no competitor. (C) Transcriptional activity of salmon TRβ. pSG5 or pSG5-TRβ vectors were transfected together with an xDR4 Luc reporter construct, and the indicated amount of T3 was added to the cell culture. -, No hormone.

The amino acid conservation observed between fish TRs and mammalian TRs is striking in comparison with other TRs. No doubt these receptors should have, in all these species, the same mode of action as that in tetrapods, i.e. the binding of DNA on TREs as well as the binding of T3. This has been confirmed by the characterization of salmon TR β (see below).

For flatfish, we notice the presence of a Ser-to-Asn mutation at position 240 which is known to interact directly with the ligand. We know that flatfish respond very well to T3 and that it is apparently TR α and not TR β that is up-regulated during metamorphosis (Yamano & Miwa 1998). Thus we suspect that this TR α will be fully functional, though this remains to be tested.

The RT-PCR fragments cloned are large enough to allow us to construct a phylogenetic tree showing the strong conservation between these receptors during evolution. As already observed in other vertebrates, TR α appears to accumulate mutations over time faster than does TR β . The main result of the phylogenetic tree is that TR β genes follow classical fish phylogeny, whereas phylogenetic information from TR α appears unclear (Nelson 1984, Bernardi *et al.* 1993). It is tempting to link this observation to the apparent gene duplication that arose specifically in some fish (*Salmo*, *Paralichthys*) for TR α but not TR β . A gene duplication, by allowing the sequence to accumulate more mutations because the selective pressure is reduced, may give rise to rate heterogeneity between sequences that may result in artefactual phylogeny. Nevertheless, in our TR α sequence data we did not observe any bias in the evolutionary rate that might be indicative of such an event.

Another question raised by the existence of these duplications is their distribution in various orders of teleosts. It has recently been proposed (on the basis of Hox gene-complex analysis in pufferfish and zebrafish, as well as on the basis of the observation of several paralogous sequences present in zebrafish) that early on during teleost evolution the entire genome was duplicated, resulting in a sudden increase in gene number (Amores *et al.* 1998, Wittbrodt *et al.* 1998). According to this model, the resulting copies may have been lost or conserved in various phyla depending on their function (or even randomly). This model is reminiscent of the model of gene duplications arising very early during vertebrate evolution and giving rise to the various vertebrate paralogues (such as TR α and TR β). The data we obtained for TR α do not support this model. Effectively, we found two copies of TR α in two distinct orders of fish, but these sequences are not grouped together in a manner reminiscent of an

ancient duplication. From the tree obtained with TR β , it seems obvious that this gene has escaped this duplication event or that one of the resulting copies was lost very early on, since we obtained a phylogeny consistent with the existence of only one gene. In order to obtain information for that model we will have to study an increased taxonomic range of species containing several TR α sequences to see if an evolutionary pattern indicative of an ancient gene-duplication event becomes obvious.

New isoforms of TR α and TR β

We identified several isoforms in teleost fish that were unknown in tetrapods. For TR α , we found an in-frame deletion of the exon homologous to exon 7 of the human TR α gene that results in a deletion of 49 amino acids localized in the LBD. Surprisingly, we isolated the same isoform, in *Ambystoma mexicanum* as well as in the newt *Pleurodeles waltii* (R Safi & V Laudet, unpublished observations). Since this isoform contains a deletion in the LBD, including the region encoding for the major part of the ligand binding pocket, it is likely that it will not bind the ligand but will still bind to DNA. Thus, we predict that this isoform will behave as a dominant negative regulator of the function of TRs like the human TR α 2 isoform (Koenig *et al.* 1989, Katz & Lazar 1993). In this context, it is important to note that the transcript corresponding to this isoform is expressed in tilapia, suggesting a functional role *in vivo*.

For TR β , we found that the small teleost-specific nine-amino-acid insertion is in fact an alternative, since in salmon we found two types of transcripts, with or without this region, and the same phenomenon was observed in the flounder (Yamano & Inui 1995). This insertion, which may correspond to an exon (see Fig. 2C), is located between H2 and H3 in a region forming a loop in the three-dimensional structure. It is difficult to predict the effect of the presence of these amino acids on the function of the receptor. From the data obtained in the functional characterization of the *Salmo* TR β , it is clear that the presence of these amino acids does not impair the basic function of the receptor (see below).

Functional characterization of salmon TR β

The large TR β isoform of salmon was tested in gel-shift experiments and transactivation assays to check if this insertion can influence its ability to bind DNA and transactivate (Fig. 6). We found that it can function as a classical receptor in all of the response elements we tested, except one. Indeed,

binding of salmon TR β to the TRE found in the MBP promoter is very weak.

Our transactivation experiments suggest that the *Salmo* TR β binds T3 with an affinity comparable to that of amphibian or mammalian receptors, since we observed a convincing dose/response curve, with a minimal transactivation for 1 nM T3, which is in accordance with the K_d for other TRs (Fig. 6C). This suggests that the nine-amino-acid insertion does not have a major impact on T3 binding affinity or on the transactivation potential of the receptor. Interestingly, we observed a clear transcriptional repression in the absence of hormone, suggesting that *Salmo* TR β in the apo-form is a repressor, which is similar to the known situation for mammalian and amphibian TR β (Hörlein *et al.* 1995, Chen & Evans 1995). Taken together, these data suggest that *Salmo* TR β binds to co-repressors and co-activators, like its mammalian homologs.

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