Differential expression of thyroid hormone receptor isoforms is strikingly related to cardiac and skeletal muscle phenotype during postnatal development

P White and M J Dauncey
The Babraham Institute, Cambridge CB2 4AT, UK
(Requests for offprints should be addressed to M J Dauncey; E-mail: joy.dauncey@bbsrc.ac.uk)

ABSTRACT

The genomic actions of thyroid hormones (THs) are mediated by receptors (TRs) that are encoded by two protooncogenes, c-erbA-α and c-erbA-β. The precise functions of the TR isoforms are unclear and this study focuses on the potential roles of the TRα and TRβ isoforms in mammalian striated muscles postnatally. The porcine TRα1, TRα2 and TRβ1 cDNAs were first cloned, sequenced and characterised by Northern blotting. A quantitative analysis of TR isoform expression was then undertaken, using RNase protection analysis with novel riboprobes designed to detect relative expression levels of TRα1, TRα2, TRβ1 and TRβ2, in functionally distinct muscles from 7-week-old pigs kept under controlled conditions of nutrition and thermal environment. We found a striking musclespecific pattern of TRα isoform distribution: in heart the mRNA level of TRα2 (non-TH binding) was markedly greater (P<0.01) than that of TRα1 (TH binding); in longissimus dorsi the opposite pattern of expression occurred (TRα1>TRα2, P<0.001); in soleus, diaphragm and rhomboideus there were no differences between the two isoforms. The overall abundance of TRβ was very much lower than that of TRα, and TRβ1 was expressed at a higher level than TRβ2 in all muscles. Together with recent data from TR gene inactivation studies and the established role of TH in determining myosin heavy chain isoform expression and muscle phenotype, these results suggest a role for differential expression of TR isoforms in acquisition and maintenance of optimal cardiac and skeletal muscle function.

Journal of Molecular Endocrinology (1999) 23, 241–254

INTRODUCTION

Thyroid hormones (THs: l-tri-iodothyronine, T₃; l-tetra-iodothyronine, thyroxine, T₄) play central roles in the control of growth, development and metabolism (Oppenheimer et al. 1987, Dauncey 1990, Chin & Yen 1997) and they are essential for normal development and function of striated muscle (Muscat et al. 1995). Coincident with a perinatal increase in circulating TH levels, embryonic and neonatal myosin heavy chain (MyHC) isoforms are repressed and adult MyHC isoforms are accumulated, and there are also marked muscle-specific changes in cellular metabolism and mitochondrial biogenesis (d’Albis & Butler-Browne 1993, Herpin et al. 1996). Postnatally, THs continue to influence muscle phenotype, changing transcription levels of myogenic regulatory factors, altering membrane-bound ATPases and metabolic properties, and inducing switching from type I slow to type II fast MyHC, with the extent of these changes being dependent on muscle type (Izumo et al. 1986, Dauncey & Harrison 1996, Harrison et al. 1996b, 1997).

THs act at many sites within the cell (Davis & Davis 1997), and their major influence is at the genomic level via a group of nuclear receptors (TRs) which regulate the expression of numerous target genes (Green & Chambon 1986, Munoz & Bernal 1997). The TRα gene (c-erbA-α) produces an RNA transcript that is alternatively spliced to produce the carboxyl terminal variants TRα1, TRα2 and, in mice, TRα3, while the TRβ gene (c-erbA-β) produces two amino terminal variants, TRβ1 and TRβ2 (Lazar 1993). The TRα1, TRβ1 and TRβ2 isoforms can bind TH and transactivate TH response elements (TREs) on target genes. However, TRα2 cannot bind TH because of structural changes in the ligand binding domain (LBD). Although this results in reduced DNA binding affinity, TRα2 is thought to compete for TREs and
to act as a dominant negative regulator of TH activity (Yen & Chin 1994, Yang et al. 1996).

Nuclear TH binding studies in porcine tissues indicate that perinatal TR ontogeny is different in skeletal muscle compared with liver (Duchamp et al. 1994), and that energy status plays a key role in regulating muscle TRs postnataally (Morovat & Dauncey 1995). However, the precise functions of the individual TR isoforms are unclear. Gene in activations of TRα1, TRα1/α2 and TRβ in mice suggest that differential expression of TR isoforms in developing and adult tissues reflects differences in function (Hsu & Brent 1998). Despite the profound influences of THs on different muscle types, there is, however, virtually no information on TR isoform expression in functionally distinct muscles.

The aims of the present study were to clone and characterise the cDNAs encoding the porcine TR isoforms, and to determine the relative expression of these isoforms in functionally distinct skeletal and cardiac muscles during postnatal development. The focus was on porcine TRs because the young pig makes a particularly good model for the human infant in relation to many developmental, metabolic, endocrine and physiological characteristics. We describe a novel method for quantifying TR isoform mRNAs, using riboprobes designed to detect both TRα isoforms and both TRβ isoforms simultaneously. Our findings highlight distinct muscle-specific differential expression patterns of the TR isoforms and suggest key roles for these isoforms in the acquisition and maintenance of optimal muscle function postnataally.

**MATERIALS AND METHODS**

**Animals and tissues**

Previous studies have demonstrated that energy status profoundly affects nuclear TH binding capacity (Morovat & Dauncey 1995), and that food intake influences TR isoform expression (Bakker et al. 1998). All tissues were therefore obtained from animals kept under carefully controlled conditions of food intake and thermal environment. Five male pigs (Sus scrofa) of the Large White breed were obtained at 3 weeks of age and housed at a temperature close to thermal neutrality (26 °C). For the next 4 weeks, the animals were provided with a controlled amount of food that enabled optimal growth. Because several hormonal and metabolic parameters are influenced by the time of the last meal (Dauncey et al. 1994, Morovat & Dauncey 1998), tissue sampling was carried out 20 h after feeding. At 7 weeks, the animals were sedated and then killed with sodium pentobarbitone as described previously (Harrison et al. 1996a). The following tissues were dissected rapidly, divided into 1–5 g portions, frozen in liquid nitrogen and stored at −70 °C: cardiac ventricular muscle, soleus, diaphragm, rhomboideus, longissimus dorsi, liver, thyroid gland and cerebrum.

**RNA isolation**

Total RNA was extracted from 0.5 g portions of tissue using a method based on the guanidine thiocyanate method of Chomczynski & Sacchi (1987). The final RNA pellet was dissolved in 0.3 M sodium acetate, pH 5.2 and quantified by duplicate absorbance readings at 260 nm. A constant relation between A260 and poly(A)+ content was found for RNA from all tissues. RNA samples were stored in ethanol at −70 °C.

**Cloning**

First-strand cDNA was synthesised from 1 µg heart total RNA using an oligo(dT) primer in combination with a genetically engineered RNase H– Moloney murine leukaemia virus reverse transcriptase (SuperScript II RNase H– MMLV-RT, Gibco BRL, Paisley, Strathclyde, UK). This enzyme is reported to perform reverse transcription more faithfully than other RT enzymes while not degrading RNA, thus giving higher yields of cDNA and more full-length product (Kotewicz et al. 1988). Oligonucleotide primer pairs were designed according to the published human TRα1 (Laudet et al. 1991), TRα2 (Nakai et al. 1988) and TRβ (Weinberger et al. 1986) sequences (Accession Numbers X55005, J03239 and X04707 for α1, α2 and β1 respectively). The polymerase chain reaction (PCR) was carried out using the heart cDNA as a template to generate both TRα and TRβ DNA fragments as indicated in Fig. 1. To ensure that the PCR amplification produced DNA with a sequence true to that of the template, a high fidelity DNA polymerase system was used (Expand High Fidelity PCR System, Boehringer Mannheim, Lewes, East Sussex, UK). The inherent 3'-5' exonuclease proof-reading activity of the Pwo DNA polymerase contained in this system resulted in high fidelity DNA synthesis (Barnes 1994). Amplified DNA fragments were purified and cloned into pGEM-T Easy plasmid vector (Promega, Southampton, Hants, UK). These plasmids were then used as templates for automated fluorescent sequencing using an Applied Biosystem Prism 377 DNA sequencer.
1. Schematic representation of thyroid hormone receptor (TR) isoform structures, based on deduced amino acid sequences from cloned porcine cDNAs for TRα (Fig. 2) and those of human for TRβ. Colours represent the functional domains common to all nuclear hormone receptors: green, amino terminal transactivation domain (A/B); red, DNA binding domain (C); blue, ligand binding domain, consisting of hinge region (D), and carboxy ligand binding and dimerisation domains (E/F). Numbering of amino acid residues is indicated above the domains. The two TRα variants are homologous from the 5' end to amino acid 370; after this point the sequences diverge, as represented by the light blue of the TRα2 variant. The two TRβ isoforms differ in their N-terminal section, as indicated by the lighter green of the TRβ2 variant. Coding region is flanked by two thick black lines representing 5' and 3' untranslated regions. Thin black lines underneath each structure show positions of overlapping cDNA clones used to sequence the variants. Arrows denote location of primers designed according to the human TRα (H1, H2, H3) and TRβ (H4, H5) sequences used in PCR amplification of the porcine TR cDNAs. Red lines denote probe sequences: thin lines represent the three cDNA fragments used in Northern blot analyses; thick lines represent riboprobes used in RNase protection analyses. The TRα riboprobe was 100% homologous to TRα1 and 70% homologous to TRα2, giving 218 and 153 bp protection products respectively. The TRβ riboprobe was 100% homologous to TRβ1 and 67% homologous to TRβ2, giving 230 and 154 bp protection products respectively.

FIGURE 1. Schematic representation of thyroid hormone receptor (TR) isoform structures, based on deduced amino acid sequences from cloned porcine cDNAs for TRα (Fig. 2) and those of human for TRβ. Colours represent the functional domains common to all nuclear hormone receptors: green, amino terminal transactivation domain (A/B); red, DNA binding domain (C); blue, ligand binding domain, consisting of hinge region (D), and carboxy ligand binding and dimerisation domains (E/F). Numbering of amino acid residues is indicated above the domains. The two TRα variants are homologous from the 5' end to amino acid 370; after this point the sequences diverge, as represented by the light blue of the TRα2 variant. The two TRβ isoforms differ in their N-terminal section, as indicated by the lighter green of the TRβ2 variant. Coding region is flanked by two thick black lines representing 5' and 3' untranslated regions. Thin black lines underneath each structure show positions of overlapping cDNA clones used to sequence the variants. Arrows denote location of primers designed according to the human TRα (H1, H2, H3) and TRβ (H4, H5) sequences used in PCR amplification of the porcine TR cDNAs. Red lines denote probe sequences: thin lines represent the three cDNA fragments used in Northern blot analyses; thick lines represent riboprobes used in RNase protection analyses. The TRα riboprobe was 100% homologous to TRα1 and 70% homologous to TRα2, giving 218 and 153 bp protection products respectively. The TRβ riboprobe was 100% homologous to TRβ1 and 67% homologous to TRβ2, giving 230 and 154 bp protection products respectively.
Initially, several overlapping fragments were cloned (Fig. 1), ranging in size from 500 to 1500 bp. The sequences of these smaller fragments were combined to give complete cDNA sequences for TRα1 and TRα2, and a partial cDNA sequence for TRβ1. To verify the sequence data, the complete TRα1 and TRα2 cDNAs were cloned as two single PCR products. This was achieved using primers designed within the 5’ and 3’ untranslated regions (UTR) of the TRα1 and TRα2 mRNAs. For TRα1, the 5’ primer, H1 (5’-TGTGAAAGGCCCAGTG CTG-3’), comprised nucleotides −155 to −137 of the human TRα1 sequence. The 3’ TRα1-specific primer, H2 (5’-GCCTGGTTTCCAAAGTGCA-3’), comprised nucleotides 1755 to 1773 of the human TRα1 sequence. For TRα2, primer H1 was used with the 3’ TRα2-specific primer, H3 (5’-ACAGACTCATGCCCCACT-3’), complementary to nucleotides 1842 to 1860. The largest fragment of the porcine TRβ1 cDNA was produced with the TRβ1-specific 5’ primer, H4 (5’-AGC TTGGGACAAACCGAAG-3’), which comprised nucleotides 21 to 478 of the porcine TRβ1 sequence, and the 3’ TRβ1 primer, H5 (5’-CTGTGGGCCTC TGGCTTGTG-3’), complementary to nucleotides 613 to 631 of the human TRβ1 sequence.

To exclude completely the possibility that either the DNA polymerase or the sequencing run had generated variations in nucleotide sequence, at least two clones were generated from independently reverse transcribed cDNA samples from heart RNA extracted from two different animals. Sequencing was carried out in both the sense and anti-sense directions for every DNA fragment cloned.

Northern blot analysis

Northern blot analysis was used to characterise the mRNAs encoding the porcine TRα isoforms. Samples of total RNA (20 µg) extracted from each tissue were denatured at 85 °C for 3 min in buffer containing 2·2 M formaldehyde, 50% formamide, 33 µg/ml ethidium bromide, and 1 × MOPS buffer (20 mM 3-[N-morpholino]propanesulphonic acid, 5 mM sodium acetate, 1 mM EDTA). The RNA transcripts were separated by electrophoresis in a denaturing formaldehyde gel (0·22 M formaldehyde, 0·9% agarose) in 1 × MOPS buffer. Ethidium bromide staining confirmed equal loading of RNA per lane and all samples were run against an RNA ladder for size comparison (Gibco BRL). The separated RNA was transferred to a nylon membrane (Hybond-N, Amersham International, Amersham, Bucks, UK) using a pressure blotter (PosiBlot, Stratagene, Cambridge, Cambs, UK).

Three double-stranded DNA probes (Fig. 1) were generated using PCR. The first, a 458 bp TRα1-specific probe, corresponded to nucleotides 1332 to 1773 of the porcine TRα1 coding sequence (cds). The second was a 623 bp TRα2-specific probe, corresponding to nucleotides 1294 to 1806 of the porcine TRα2cds. The third was a 458 bp TRβ1 and β2 common probe, corresponding to nucleotides 21 to 478 of the porcine TRβ1cds. Each of these DNA fragments was labelled with [α-32P]dCTP using a random priming labelling kit (Rediprime DNA labelling system, Amersham International). Blots were pre-hybridised at 65 °C in commercially available hybridisation buffer (Rapidhyb buffer, Amersham International) for 1 h. The pre-hybridisation buffer was then replaced with fresh buffer containing ~2 ng/ml of the appropriate radiolabelled DNA probe, and left to hybridise for 4 h at 65 °C. Blots were washed in 0·1 × SSC (15 mM sodium chloride, 1·5 mM sodium citrate, pH 7·0) and 0·1% SDS at 65 °C for 2 × 10 min, before autoradiography.

Construction of TR riboprobes and RNase protection analysis

RNase protection analysis was used in detailed expression studies of TR isoform abundance in functionally distinct muscles. When optimised, this is an extremely sensitive method for the detection and quantification of specific RNAs in a complex mixture of total cellular RNA, and the reaction can be 10- to 100+-fold more sensitive than Northern bloting (Melton et al. 1984). Using the newly established porcine TRα and TRβ cDNA sequences, two probes were designed for detection of expression levels of all four TR isoforms (Fig. 1). The TRα probe was generated by PCR using oligonucleotide primers as follows: the 5’ primer, 5’-AGGCTGTGCTGTGCTAATGTCA-3’, comprised the sequence between nucleotides 958 and 977; the 3’ primer, 5’-GCACCTGACTTTTCTGATGGAATAGGATGGA-3’, was complementary to nucleotides 1156 to 1175. PCR was carried out using the complete porcine TRα1 cDNA as a template. The resulting 218 bp DNA fragment was blunt-end cloned into an EcoRV linearised plasmid (pBluescript KS (+/-), Stratagene). DNA sequencing revealed that the DNA fragment had been cloned in the sense direction. For TRβ, the 5’ primer, 5’-CCATCTGGACCAACGACATGATGGAATAGGATGGA-3’, was complementary to nucleotides 400 to 418. PCR was carried out using the cloned fragment of the porcine TRβ1 cDNA as a template.
The resulting 230 bp DNA was similarly cloned into pBluescript KS (+/−) and DNA sequencing revealed that the DNA fragment had been cloned in the antisense direction.

Antisense riboprobes were generated by linearising the plasmid by digestion with HindIII or EcoRI and then transcribing with T7 or T3 RNA polymerase, for TRα and TRβ respectively, in the presence of [α-32P]UTP (Amersham International). The TRα riboprobe had a full-length transcript of 295 bp, of which 218 bp hybridised fully to TRα1, corresponding to nucleotides 958 to 1175. Since this probe spanned the carboxyl terminal region, where TRα1 and TRα2 have differences in the LBD, a second protection product of 154 bp was formed, corresponding to nucleotides 265 to 418 of TRβ2. Thus, these single probes allowed accurate measurement of the expression levels of both TRα isoforms and both TRβ isoforms within a single assay.

Assays were carried out on 50 µg total RNA, extracted from heart, soleus, diaphragm, semitendinosus, longissimus dorsi and liver, using methods similar to those described previously (Dauncey et al. 1994). Samples were hybridised with a small molar excess of the radiolabelled TR riboprobe, as determined by titration analysis, to ensure linearity of the assay with respect to RNA. After 16 h hybridisation at 45 °C excess non-protected RNA was digested with RNase A (50 µg/ml ~1 U/sample; Sigma) and RNase T1 (300 U/ml ~80 U/sample; Sigma). The protected hybridisation products were purified by extraction in phenol: chloroform:isoamyl alcohol (25:24:1) and analysed by polyacrylamide–urea gel electrophoresis, followed by autoradiography. Expression levels of the two TR isoforms were quantified by image analysis, with values being expressed as optical density units. Significance of differences was tested using Student’s paired t-test.

RESULTS

Sequences of the porcine TR isoforms

In addition to several smaller overlapping gene fragments, two complete cDNAs encoding the porcine TRα1 and TRα2 receptor variants, and a partial cDNA sequence for TRβ1 were successfully cloned and sequenced (EMBL Nucleotide Sequence Database Accession Numbers AJ005797, AJ005798 and AJ238614 respectively). Due to the stringent and thorough cloning strategy employed, the authors are certain that the sequences are accurate and that no errors have been introduced as a result of DNA polymerase activity. In total the following were cloned: 1906 bp of the porcine TRα1 cDNA, corresponding to bases −136 to 1770; 1977 bp of the TRα2 cDNA corresponding to bases −136 to 1898; and 611 bases of the TRβ1, homologous to nucleotides 21 to 631 of the human TRβ1 coding region (Weinberger et al. 1986). For TRα1, the clone comprised the entire pig TRα1 cds, 136 bp of 5' UTR, and 553 bp of 3' UTR. For TRα2, the clone comprised the entire pig TRα2 cds, 136 bp of 5' UTR, and 370 bp of 3' UTR. The TRα1 and TRα2 open reading frames encode proteins of 410 and 506 amino acids (Fig. 2), with predicted average molecular masses of 46.8 and 56.3 kDa respectively.

Database searching with these sequences revealed them to be unique, with highest DNA identity (>95%) to the appropriate human sequences (Weinberger et al. 1986, Nakai et al. 1988, Laudet et al. 1991). The predicted amino acid sequences of the porcine TRα cDNAs were used to produce multiple sequence alignments with other known TRα peptide sequences (Fig. 2). A progressive, pair-wise alignment was carried out using the program PileUp (part of Wisconsin Package Version 9-0-UNIX; Genetics Computer Group (GCG) Inc., Madison, Wisconsin, USA), which uses an alignment method similar to that described previously (Higgins & Sharp 1989). Figure 2a shows the results for alignment of the TRα1 peptides. The human amino acid sequence was most similar to pig, with only one conservative amino acid substitution at position 170 (isoleucine to valine), followed by sheep (Ovis aries) (Tucker & Polk 1996), rat (Rattus norvegicus) (Thompson et al. 1987), mouse (Mus musculus) (Masuda et al. 1990), chicken (Gallus gallus) (Sap et al. 1986), Muscovy duck (Cairina moschata) (Lachuer et al. 1996), bull frog (Rana catesbeiana) (Schneider et al. 1993), African clawed frog (Xenopus laevis) (Brooks et al. 1989), Japanese flounder (Paralichthys olivaceus) (Yamano et al. 1994) and zebrafish (Danio rerio) (Essner et al. 1997). The TRα2 cDNA has been cloned completely in only two other species, human and rat (Lazar et al. 1988), and partially in the mouse (Prost et al. 1988); the results of alignment of these sequences with the pig are shown in Fig. 2b. The human TRα2 peptide was most homologous to pig, followed by rat and mouse. All three complete TRα2 peptide sequences showed a high level of variation in the C-terminal region of the LBD,
resulting in the pig TRα2 peptide being longer (506 amino acids) than that of human (490 amino acids) and rat (492 amino acids). The lower level of homology in the last 30 amino acids of the porcine TRα2 sequence, compared with human and rat, might possibly suggest that this isoform is another as yet uncharacterised splicing variant. However, with the lack of sequence data available for TRα2 in other species, this conclusion cannot be substantiated. A much more likely conclusion, based on the extremely high level of identity over the entire length of this sequence and the human and rat TRα2 cDNAs, is that is indeed TRα2.

Alignment of the TRα1 peptide sequences allowed us to produce a phylogenetic tree showing relationships between the TRα1 protein in different species (Fig. 3). A distance matrix was calculated and corrected for multiple substitutions at a single site using the Jukes-Cantor method (using the GCG 9 programme, Distance). The phylogenetic tree was reconstructed from this distance matrix using the unweighted pair group method and arithmetic averages (UPGMA; using the GCG 9 programme, GrowTree). The tree clearly shows a particularly high level of homology between human and porcine TRα1 isoforms.

**Characterisation of porcine TR mRNAs**

Northern blotting of RNA prepared from several tissues demonstrated the presence of three TRα mRNA species and four TRβ mRNA species (Fig. 4). The TRα1 specific probe hybridised to 5·3 and 7·4 kb transcripts, while the TRα2-specific probe hybridised to 2·5 and 7·4 kb transcripts. Therefore, the 5·3 kb transcript encodes the α1 variant, and the 2·5 kb transcript the non-TH binding α2 variant. The 7·4 kb transcript was detected by both probes, suggesting that it contains sequences encoding the unique 3′ ends of TRα1 and TRα2 mRNAs. The TRβ Northern probe hybridised to a large mRNA species of 7·4 kb; several smaller transcripts of 3·9, 2·5 and 1·5 kb were also expressed, but at a much lower level than the 7·4 kb transcript. The TRβ mRNA transcripts appeared to be expressed at a much lower level than the TRα transcripts, since autoradiography was for 10 days with the TRβ probe but only 3 days with the TRα probes.

Figure 4 shows that diaphragm and *rhomboideus* expressed the highest level of the TRα1 transcript, while heart and cerebrum expressed the highest level of TRα2; the 7·4 kb TRα transcript was expressed at the greatest level in heart. All three TRα transcripts were expressed at their lowest level in liver, and were also relatively low in the thyroid gland. Expression of the 7·4 kb TRβ transcript was highest in heart, followed by diaphragm, *rhomboideus*, liver and brain. *Soleus* and *longissimus dorsi* had low expression levels of TRβ, and the transcript was barely detectable in thyroid gland.

**Tissue-specific expression of the porcine TRα and TRβ isoforms**

By contrast with Northern blotting, RNase protection gave a considerably more detailed expression analysis of all four TR isoforms. The TRα riboprobe had been designed to detect the α1 and α2 isoforms simultaneously (Fig. 1), giving protected hybridisation products of 218 and 153 bp respectively. Similarly, the TRβ riboprobe gave protected hybridisation products of 230 and 154 bp for β1 and β2 respectively. The proportions of [α32P]UTP incorporated into each hybridisation product were calculated and these ratios were used to correct the optical density values. In this way, the relative levels of TR isoform expression could be determined both between and within individual tissues.

For all tissues, expression of the TRα isoforms was considerably greater than that of the TRβ isoforms. RNase protections using the TRα riboprobe required overnight exposure to X-ray film, whereas those with the TRβ riboprobe required exposure for 3 to 5 days. This difference in expression of TRα compared with TRβ is illustrated in Fig. 5 for heart and *longissimus dorsi*.

Major differences were observed with respect to the relative abundance of the four TR isoforms both between and within tissues (Figs 6 and 7). Expression of TRα1 was similar in all muscles, whereas TRα2 showed a very much more variable pattern of expression (Fig. 6). The expression of TRα2 was extremely high in heart, and more than double that in *soleus*, diaphragm, and *rhomboideus*. By contrast, TRα2 expression in *longissimus dorsi* was less than half that in the other skeletal muscles.
and less than one fifth of that in heart. Expression of the two TRα isoforms was lowest in liver.

Particularly striking were the tissue-specific differences in relative levels of TRα isoforms within each muscle. In heart, the relative level of α2 was more than twice that of α1 ($P<0.01$), whereas in soleus, diaphragm and rhomboideus there were no significant differences between the two isoforms. However, longissimus dorsi and liver showed the opposite pattern of expression, with levels of α1 being twice as great as α2 ($P<0.001$).

Although TRβ isoform expression was considerably less than TRα isoform expression, a
tissue-specific pattern of distribution was again seen for these isoforms (Fig. 7). At 7 weeks postnatally the levels of TR$\alpha_1$ mRNA were highest in heart, intermediate in soleus, diaphragm, rhomboideus and liver, and lowest in longissimus dorsi. The tissue-specific pattern of TR$\beta_2$ expression was similar to that of TR$\beta_1$, although for all tissues examined expression of TR$\beta_2$ was significantly lower than that of TR$\beta_1$.

DISCUSSION

We have successfully cloned and characterised for the first time the cDNAs encoding the porcine TR isoforms. Multiple sequence alignment and phylogenetic analysis demonstrated that the porcine TR sequences were most similar to those of human, supporting the use of the young pig as a particularly good biological model for the human infant. Detailed expression analysis using RNase protection demonstrated distinct and previously unreported patterns of TR isoform expression in a range of functionally distinct muscles.

The sequences of the TR$\alpha$ variants showed very high homology in a group of divergent species ranging from fish to man. The highest level of homology was seen in the DNA binding domain (DBD), followed by the ligand binding domain (LBD), then the hinge domain. The lowest level of homology was seen in the N-terminal A/B domain. This is consistent with the modular organisation of the majority of nuclear receptors, in which DBDs and LBDs are conserved in all family members with only a few exceptions (Laudet 1997). The fact that these receptors have been so highly conserved during evolution clearly indicates the important functional roles of both the TR$\alpha$ isoforms. Gene knockout studies on TR$\alpha$ in mice demonstrate some of these functions during postnatal development (Hsu & Brent 1998). Abrogation of TR$\alpha_1$ and TR$\alpha_2$ results in severe developmental problems, including growth arrest, incomplete maturation of the small intestine and especially the smooth muscle layers, impaired bone development, hypothyroidism and death within 5 weeks of birth (Fraichard et al. 1997).

Use of TR$\alpha$ isoform-specific probes showed that in the pig, mRNA transcripts of 5·3 and 2·5 kb encode TR$\alpha_1$ and TR$\alpha_2$ respectively. The human TR$\alpha_1$ protein is translated from mRNA of 5·0 kb which contains a lengthy 3' UTR, a feature common among nuclear receptor genes; the TR$\alpha_2$ protein is translated from mRNA of 2·7 kb. These similarities in size between the two species suggest that the genomic organisation of the porcine c-erbA-$\alpha$ gene is similar to that of human, in which the TR$\alpha$ gene is split into 10 exons distributed along 27 kb of chromosome 17 (Laudet et al. 1991). In our study, both probes also revealed the presence of a third transcript of 7·4 kb, the abundance of which followed an expression pattern similar to that of the TR$\alpha_2$ transcript: highest level in heart, lowest in liver. The presence of a similarly large transcript has been reported in mouse (6·4 kb) (Wood et al. 1991) and rat (6·6 kb) (Murray et al. 1988), and may represent precursor mRNAs from which the smaller transcripts are generated.

The pattern of TR$\beta$ mRNA transcript expression was markedly different from that of TR$\alpha$. Use of a probe designed to detect transcripts encoding both TR$\beta$ isoforms revealed that this gene is expressed in several tissues as an abundant 7·4 kb mRNA and several smaller, less abundant transcripts of 3·9, 2·5 and 1·5 kb. As with TR$\alpha$, this pattern of mRNA expression is very similar to the human: an abundant transcript of 10 kb and several smaller, less abundant transcripts of 5, 3 and 2 kb have been
reported (Sakurai et al. 1989). It was suggested that the smaller transcripts could be degradation products of the 10 kb mRNA. However, the results of the current study would suggest that they represent alternative TRβ mRNAs. Since the probe detected both isoforms it is not possible to say whether these transcripts represent the different isoforms.

An indication of the distribution of TR isoforms in various tissues was first obtained by Northern blotting, but this did not provide quantitative data nor did it allow assessment of relative distribution of the isoforms within a tissue. This problem was overcome by the use of rigorously optimised RNase protection analysis (White & Dauncey 1998). Particularly striking was the finding that cardiac tissue expresses a very high level of the non-TH binding α2 variant compared with the level of TRα1, whereas in longissimus dorsi the opposite pattern of distribution occurs, with TRα1 being the predominant isoform. The expression of TRβ1 was greater than TRβ2 in all tissues and relative levels of each isoform were muscle specific. However, the functional significance of these differences in TRβ is unclear because TRβ expression was considerably lower than that of TRα, suggesting that in muscle these isoforms may maintain a level of basal responsiveness to TH. The high sensitivity of the

![Image](image_url)
RNase protection assay allowed detection and quantification of extremely low levels of TRβ2 mRNA in all the tissues investigated.

The findings on muscle-specific differential expression of TRα isoforms are relevant to our understanding of the acquisition of optimal muscle function. It has been speculated that the differing responses of various skeletal muscles to TH are due to differences in total TRs, muscles with a higher TR number being more sensitive to the effects of TH (d’Albis & Butler-Browne 1993). Although the relation between observed differences in TR isoform mRNAs and expression of TR isoform protein remains to be established, our findings suggest that it is the pattern of TR isoform expression, rather than total TRs, that may regulate muscle phenotype. The TRα2 variant cannot actively inhibit transcription (Tagami et al. 1998) but its dominant negative activity will protect the tissue from the action of TH by competing with TRα1 for TRE binding. By this definition, of the muscles examined longissimus dorsi will be the most sensitive and heart the least sensitive to the action of TH. In view of the role of TH in inducing switching from type I slow MyHC to type II fast MyHC (Izumo et al. 1986), in muscles where TRα1 predominates over TRα2 the majority of myofibres should express fast MyHC. By contrast, in muscles where TRα2 predominates, the majority of myofibres should express mainly slow MyHC. This hypothesis fits closely with the present findings. In 7-week-old pigs kept under the same conditions as those of the present study, longissimus dorsi contains approximately 90% fast MyHC, the other skeletal muscles examined have similar proportions of fast and slow MyHC, whereas heart contains only slow MyHC (Harrison et al. 1996a, P White, D Cattaneo and M J Dauncey, unpublished observations). Thus, the potential for rapid movement by longissimus dorsi will be accentuated by its relatively high level of TRα1, and the maintenance of slow sustained contractility by the heart will be enhanced by its high level of TRα2.

The present results are particularly relevant to our understanding of the roles of THs and TRs in regulating cardiac function postnataally. In mice lacking TRα1 because of a null mutation of the TRα1 locus, an abnormally low heart rate was observed (Wikstrom et al. 1998). The authors could not explain the mechanism involved because none of the well-known TR target genes in the heart such as Ca²⁺-ATPase, Na⁺,K⁺-ATPase and β-adrenergic
receptors were dysregulated. However, cardiac MyHC expression was not investigated. In humans, excessive production of THs has a severe impact on cardiac function, with symptoms including increased blood flow and cardiac output (Woebner 1992); the opposite effects occur with TH deficiency. These TH-induced changes in contractility are dependent on the relative amounts of α-MyHC and β-MyHC gene products expressed in cardiac tissue (Brent 1994). The products of the two cardiac MyHC chain genes dimerise to form three MyHC isoforms: α/α, α/β and β/β. Hearts rich in α-MyHC have a high intrinsic contractility state, due to the higher ATPase activity of this protein, while a preponderance of β-MyHC is associated with a greater economy of force maintenance. These genes are regulated in an antithetical manner, T3 increases the expression of α-MyHC while simultaneously decreasing that of β-MyHC (Haddad et al. 1997), and in euthyroid rats and rabbits the α/α myosin isoform predominates whereas in hypothyroid animals the β/β isoform predominates. These data in combination with the present results suggest that the balance between relative expression of TRα1 and TRα2, and the level of circulating THs, is essential in regulating cardiac MyHC expression. In the case of TRα1-deficient mice, expression of only the TRα2 variant would lead to a reduction in cardiac α-MyHC expression, and thus a reduced cardiac output.

In conclusion, our study suggests a role for TR isoforms in the regulation of muscle phenotype, with relative levels of the highly expressed TRα1 and TRα2 controlling TH action in switching between fast and slow MyHC, and the less abundant TRβ providing a basal level of TH responsiveness. Recent studies of myofibre proportions in the soleus muscle of TR knockout mice support this hypothesis (Yu et al. 1999). Abrogation of both TRα isoforms resulted in a significant upregulation of type I MyHC, while lack of TRβ did not alter MyHC composition. Our results also suggest an important role for the ratio TRα1:TRα2 in development and optimal function of the heart. The possibility that TRα isoforms have specific functions in cardiac and skeletal muscle is supported by recent studies of postnatal development in TR knockout mice, which highlight specific functions for TRα and TRβ in many tissues, and demonstrate only partial functional redundancy between these two families of TR (Gauthier et al. 1999). Future studies should focus on developmental and environmental regulation of the TR genes, and additional factors that are required for optimal TR function. Thus, TRs require heterodimerisation with retinoid X receptor (RXR) for maximal DNA binding affinity (Reginato et al. 1996). A study on the relative levels of the α, β and γ forms of the RXR family in heart and functionally distinct skeletal muscles would further elucidate the complex regulatory roles of the TR isoforms.

ACKNOWLEDGEMENTS

We thank Dr J Li for excellent advice and critical reading of the manuscript, Mr K A Burton for invaluable technical assistance and Mr J Coadwell for expert advice on sequence analysis and bioinformatics. PW was in receipt of a Medical Research Council Postgraduate Studentship. The Babraham Institute is supported by the Biotechnology and Biological Sciences Research Council.

REFERENCES


Baker O, Razaki H, de Jong J, Ris-Stalpers C & Wiersinga WM 1998 Expression of the α1, α2, and β1 T3-receptor mRNAs in the fasted rat measured using competitive PCR. Biochemical and Biophysical Research Communications 242 492–496.

Barnes WM 1994 PCR amplification of up to 35-kb DNA with high fidelity and high yield from lambda bacteriophage templates. Proceedings of the National Academy of Sciences of the USA 91 2216–2220.


Essner JJ, Breuer JJ, Essner RD, Fahrenkrug SC & Hackett PB Jr 1997 The zebrafish thyroid hormone receptor α1 is


Izumo S, Nadal-Ginard B & Mahdavi V 1986 All members of the MHC multigene family respond to thyroid hormone in a highly tissue-specific manner. *Science* **231** 597–600.


Masuda M, Yasuhara S, Yamashita M, Shibuya M & Odaka T 1990 Nucleotide sequence of the murine thyroid hormone receptor (α1) cDNA. *Nucleic Acids Research* **18** 3055.


Oppenheimer JH, Schwartz HL, Mariah CN, Kinlaw WB, Wong NC & Frecce HK 1987 Advances in our understanding of thyroid hormone action at the cellular level. *Endocrine Reviews* **8** 288–308.


Tagami T, Kopp P, Johnson W, Arseven OK & Jameson JL 1998 The thyroid hormone receptor variant α2 is a weak antagonist because it is deficient in interactions with nuclear receptor corepressors. *Endocrinology* **139** 2535–2544.


Wood WM, Ocran KW, Gordon DF & Ridgway EC 1991 Isolation and characterization of mouse complementary DNAs encoding α and β thyroid hormone receptors from thyrotrope cells: the mouse pituitary-specific β2 isoform differs at the amino terminus from the corresponding species from rat pituitary tumor cells. Molecular Endocrinology 5 1049–1061.


Yang YZ, Burgos-Trinidad M, Wu Y & Koenig RJ 1996 Thyroid hormone receptor variant α2. Role of the ninth heptad in DNA binding, heterodimerization with retinoid X receptors, and dominant negative activity. Journal of Biological Chemistry 271 28235–28242.

Yen PM & Chin WW 1994 Molecular mechanisms of dominant negative activity by nuclear hormone receptors. Molecular Endocrinology 8 1450–1454.


REVISED MANUSCRIPT RECEIVED 26 May 1999